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Biophysical Properties of Cellular Membranes in Gram-Positive Bacterial Pathogens and Their Impact on Major Physiological Attributes and Virulence Determinants

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BIOPHYSICAL PROPERTIES OF CELLULAR MEMBRANES IN GRAM-POSITIVE BACTERIAL PATHOGENS AND THEIR IMPACT ON MAJOR PHYSIOLOGICAL ATTRIBUTES AND VIRULENCE DETERMINANTS

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The cytoplasmic membrane of bacterial cells, forming an essential barrier from the surroundings, is a critical component of cellular physiology ensuring proper survival and maintenance of major cellular functions. The integrity of the membrane is an important feature that plays an essential role in the transport of solutes and nutrients through active and passive pathways, functions of membrane-associated proteins, electron transport and ATP synthesis, maintaining turgor pressure and combating environmental stresses, and thus is a crucial factor of a majority of cellular adaptations. The various biophysical properties affecting the integrity of this membrane are mainly determined by the composition and proportion of the fatty acyl residues of membrane phospholipid backbone which are subject to dynamic changes in response to the external environment to regulate the ideal fluidity/viscosity of the membrane. This enables the bacteria to adapt to changing environments. Additionally, membrane fatty acid composition has a major influence on bacterial pathogenesis and virulence, susceptibility to antimicrobials and broader aspects of bacterial physiology.

Gram-positive bacterial membranes are made mostly of varying proportions of straight-chain and branched-chain fatty acids distributed on the phosphatidyl glycerol molecules along with unsaturated fatty acids in some case. Membrane fatty acid biosynthesis that determines this lipid composition has gained attention over recent years as a novel and efficient target for therapeutic agents. A key to study the dynamics of the membrane and its relation to the bacterial physiology is to design successful tools to induce or study alterations, native or novel, to the lipid composition and analyze the resulting consequences on major cellular attributes. This thesis describes the studies of the membranes of two major gram- positive bacterial pathogens, *Listeria monocytogenes* and *Staphylococcus aureus*, both posing a threat to human health due to either unsuccessful containment or rapid antibiotic resistance acquirement. The first chapter describes the modulations in the membrane composition of *Listeria monocytogenes*, a psychrophilic bacterium, through chemical supplementation which results in novel fatty acids by non-native enzymatic reactions and the consequences on the bacterial physiology. The second chapter outlines the versatility of *Staphylococcus aureus* membrane fatty acid composition in differencing growth environments, in vitro or in vivo. The following research will contribute a better understanding of fundamental membrane biophysical parameters in these bacterial pathogens that has been the target for novel drugs for a decade now, along with providing knowledge to support the intensive search for newer and more effective infection control strategies.

KEYWORDS: Membrane fatty acid composition, membrane fluidity, physiology, virulence

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SHORT BRANCHED- CHAIN C6 CARBOXYLIC ACIDS RESULT IN INCREASED GROWTH, NOVEL 'UNNATURAL' FATTY ACIDS AND INCREASED MEMBRANE FLUIDITY IN A *LISTERIA MONOCYTOGENES* BRANCHED- CHAIN FATTY ACID-DEFICIENT MUTANT

ABSTRACT

Listeria monocytogenes is a psychrotolerant food borne pathogen, responsible for the high fatality disease listeriosis, and expensive food product recalls. Branched-chain fatty acids (BCFAs) of the membrane play a critical role in providing appropriate membrane fluidity and optimum membrane biophysics. The fatty acid composition of a BCFA-deficient mutant is characterized by high amounts of straight-chain fatty acids and even-numbered iso fatty acids, in contrast to the parent strain where odd-numbered anteiso fatty acids predominate. The presence of 2-methylbutyrate (C5) stimulated growth of the mutant at 37ºC and restored growth at 10ºC along with the content of odd-numbered anteiso fatty acids. The C6 branched-chain carboxylic acids 2-ethylbutyrate and 2-methylpentanoate also stimulated growth to a similar extent as 2-methylbutyrate. However, 3-methylpentanoate was ineffective in rescuing growth. 2-ethylbutyrate and 2-methylpentanoate led to novel major fatty acids in the lipid profile of the membrane that were identified as 12-ethyltetradecanoic acid and 12-methylpentadecanoic acid

respectively. Membrane anisotropy studies indicated that growth of strain MOR401 in the presence of these precursors increased its membrane fluidity to levels of the wild type. Cells supplemented with 2-methylpentanoate or 2-ethylbutyrate at 10ºC shortened the chain length of novel fatty acids, thus showing homeoviscous adaptation. These experiments use the mutant as a tool to modulate the membrane fatty acid compositions through synthetic precursor supplementation, and show how existing enzymes in *L. monocytogenes* adapt to exhibit non-native activity yielding unique 'unnatural' fatty acid molecules, which nevertheless possess the correct biophysical properties for proper membrane function in the BCFA-deficient mutant.

1. Introduction

Listeria monocytogenes is a Gram-positive, foodborne, intracellular pathogen that is the causative agent of listeriosis. The organism is also responsible for periodic expensive food product recalls when food is found to be contaminated with *L. monocytogenes*. Early this year, a total of 35 people were infected from a multistate *Listeria* outbreak from prepackaged Granny Smith and Gala apples. This was closely followed by a major statewide outbreak from Blue Bell creamery products followed by immediate recalls and reports that the contamination dated back several years (http://www.cdc.gov/listeria/outbreaks). The ability of *L. monocytogenes* to grow at refrigeration temperatures is an important factor in its role as a foodborne pathogen [1].

The fatty acid composition of the *L. monocytogenes* cytoplasmic membrane is unusual in that it is composed almost entirely of branched-chain fatty acids (BCFAs) [**2, 3,** 4]. Typically, the major fatty acids of the organism are anteiso C15:0, anteiso C17:0 and iso C15:0. This fatty acid composition enables *L. monocytogenes* to adapt to growth at low temperatures, mainly by increasing the content of anteiso C15:0 by a combination of fatty acid chain shortening and branched-chain switching from iso to anteiso [2, **3,** 4, 5]. This is a homeoviscous adaptation to maintain appropriate membrane fluidity [6].

BCFAs are biosynthesized from the branched-chain amino acids isoleucine (anteiso fatty acids), leucine (odd-numbered iso fatty acids) and valine (even-numbered iso fatty acids) via branched-chain amino acid transaminase and branched- chain α-keto acid dehydrogenase (Bkd) [7]. Mutants in the *bkd* gene cluster are cold-sensitive, deficient in BCFAs, and have lower membrane fluidity that the parent strain, and all these defects can be corrected with the short-branched- chain carboxylic acids (BCCAs) that act as precursors for BCFAs via a pathway that bypasses the branched- chain amino acids and Bkd [2, 5, 8, 9].

Studies with the cold-sensitive mutants, *cld-1* and *cld-2* revealed a switch in the fatty acid composition to one where the major fatty acids are straight-chain fatty acids (SCFAs) and iso-even BCFAs [2, 5, 8, 9], a fatty acid profile incompatible with good growth at low temperature due to high membrane viscosity. In the absence of branched-chain keto acid dehydrogenase activity it is proposed that the SCFAs originate from butyryl CoA, and isobutyryl CoA. Isobutyryl CoA, the precursor of even-numbered iso BCFAs, may be produced via valine dehydrogenase or isomerization of butyryl CoA [2]. However, the biophysical properties of the membrane have broader impacts in listerial physiology and pathogenicity than just cold adaptation. Giotis *et al.* [10] have shown that the BCFA-deficient *cld* mutants tolerate acidic and alkaline pH less well than the parent strain, a tolerance that can be restored by medium supplementation with 2-methyl butyrate (2-MB). Sun and O' Riordan [11] showed that BCFA-deficient mutants grew and survived less well in macrophages, exhibited decreased production of the key virulence factor listeriolysin O, and were highly attenuated in a murine model of infection. In an extension of these studies, BCFAs played a critical role in protection against antimicrobial peptides and peptidoglycan hydrolases [12]. In all these cases 2-MB restored the anteiso C15:0 and anteiso C17:0 fatty acid content and the defects in the mutants to a large extent.

In 1971 Kaneda [13] showed that various C6 BCCAs led to the production of novel fatty acids derived from these precursors in *Bacillus subtilis*. 2-ethylbutyrate (2-EB) and 2-methylpentanoate (2-MP) were the most effective precursors. These two precursors in high (100 mM) concentrations also led to the production of novel "unnatural" fatty acids when added to the cultures of wild type *L. monocytogenes* [14]. Also, Willecke and Pardee [15] studied a set of chemical analogues of natural BCFA-yielding precursors and found that a *B. subtilis bkd* mutant used them in the same pattern as noted by Kaneda (1971) [13]. However, neither of these studies

examined the effects of the C6 BCCA precursors on growth at low temperatures. It was of interest to see whether these precursors would stimulate the growth of BCFA-deficient *L. monocytogenes* mutant MOR401 at various temperatures, and see what impact they had on the fatty acid composition of the mutants and their membrane biophysical properties. 2-MP and 2-EB supplementation led to major amounts of novel fatty acids, increased membrane fluidity, and stimulated the growth of the BCFA-deficient mutant at 37°C and 10ºC.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. monocytogenes strains used in this study were parent strain 10403S and cold-sensitive mutant MOR401 (kindly provided by Yvonne Sun and Mary X. D. O'Riordan) harboring a *Tn917* transposon insertion in the *lpd* gene of the *bkd* gene cluster (lipoamide dehydrogenase, E3) created by transduction of the mutation from strain *cld-2* [2, 5] into strain 10403S [11]. The strains were grown in Brain-Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI). Starter cultures of mutant strain MOR401 were grown in medium supplemented with erythromycin (1 ug ml^{-1}).

For growth and fatty acid composition studies 50 ml of BHI medium, not supplemented with any antibiotic, in a 300 ml Erlenmeyer flask were inoculated with 2% (vol/vol) of overnight starter culture. The cells were grown at 37°C and 10°C with continuous shaking at 200 rpm in the presence of 1 mM concentrations of 2-MB and the C6 BCCAs 2-EB, 2-MP and 3-methyl pentanoate (3-MP) along with a wide range of other short BCCAs with varying chain lengths shown in Table 1. Straight chain precursor, butyrate, was used as a negative control. These BCCAs and butyrate were neutralized with 10 M NaOH to a final pH of 7.0 and added to BHI as filter sterilized solutions. The growth kinetics of the strains were monitored by measuring OD_{600} using

a Beckman DU-65 spectrophotometer. Cultures were appropriately diluted after the OD_{600} reached 0.5. Growth experiments were carried out on three separate occasions, and results of representative experiments are shown.

2.2. Membrane fatty acid analysis

Cells grown in BHI with or without the BCFA precursors were harvested in mid-exponential phase (OD_{600} 0.4-0.6) by centrifugation at 3000 x *g* at 4°C for 15 minutes, and the pellet was washed 3 times with cold sterile distilled water. The fatty acids in the bacterial cells (30 to 40 mg [wet weight]) were saponified, methylated, and extracted. The resulting methyl ester mixtures were separated using an Agilent 5890 dual-tower gas chromatograph and identified using the MIDI microbial identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc. (Newark, DE) [14, 5]. Minor fatty acids (<1% of the total) are not reported in the tables. Novel BCFAs were further characterized by electron ionization mass spectroscopy [16].

2.3. Determination of the membrane fluidity

Membrane fluidity was determined through anisotropy measurements using the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) which specifically fluoresces in the hydrophobic domain of fatty acyl chains in the lipid bilayer of the membrane [17]. Exponential phase (OD_{600} 0.4-0.6) cells grown with or without precursors were pelleted by centrifugation at 3000 x *g* at 4°C for 15 minutes and washed twice with 0.85% (wt/vol) NaCl solution. The cells were resuspended in 0.85% (wt/vol) NaCl containing 2 μM DPH (Sigma, MO) to an OD_{600} of 0.3 and incubated at 37°C for 1 hr. The resulting fluorescence polarization values of DPH were measured in a PTI fluorescence spectrophotometer using FelixGX software. Excitation of the fluorescent probe was accomplished with vertically polarized monochromatic light at 360 nm for DPH, with emission intensity

quantified at 426 nm, using a detector oriented either parallel to or perpendicular to the direction of the polarized excitation source. Lower fluidity leads to decreased movement of the probe in the membrane. This subsequently results in lesser distortion of the emitted signal and higher anisotropy values recorded by the fluorimeter.

2.4. Light microscopy

The bacterial cells were grown in BHI broth or BHI broth supplemented with BCCA precursors at 37°C and harvested at mid log phase. The pellets were washed with PBS and Gram-stained. The cells were then observed via light microscopy using differential interference contrast with the 100X objective.

3. Results

3.1. The C6 BCCAs stimulate growth of the BCFA-deficient mutant

It is well established that C5 BCCA 2-MB enhances the growth of BCFA-deficient strains of *L. monocytogenes* [2, 5]**,** and this is confirmed in Fig. 1a and 1b. The growth of the parent and MOR401 in unsupplemented BHI medium at 37°C is shown in Fig. 1a. The lag phase of strain MOR401 was significantly longer than the parent strain, the growth rate was slower and the final cell density achieved was considerably lower. As expected, 2-MB markedly stimulated the growth of the mutant at 37°C such that the growth rate and the final cell density achieved were similar to the parent strain. Interestingly the lag phase of strain MOR401 was not reduced significantly by the presence of 2-MB. C6 BCCA 2-MP had an effect on growth very similar to 2-MB, as did 2-EB except for a slightly longer period to exit lag phase with this precursor. The extended lag phase of the mutant may be related to its impairment in cell division that we noted-see Fig. 4. The precursor 3-MP had a negligible stimulating effect on growth compared to 2-MB, 2-MP or 2-EB.

Butyrate, a C4 straight-chain carboxylic acid, which can generate SCFAs [14], served as a negative control that had no stimulatory effect on growth.

At 10°C (Fig. 1b) the results were more striking. The mutant barely grew at this temperature, and growth was further diminished by inclusion of the C4 straight-chain carboxylic acid butyrate in the medium. Strikingly, 2-MP and 2-EB were equally effective if not more effective than 2-MB in stimulating growth. It is proposed that 2-MP and 2-EB act as precursors for novel unnatural fatty acids that are incorporated into the membrane [13,14,15], yet which appear to have properties that result in ideal membrane fluidity and appropriate membrane biophysical properties to allow the mutant to grow at 10°C.

3.2.Growth in the presence of C6 BCCAs results in novel membrane fatty acids

Given the growth stimulatory effects of the C6 BCCAs it seemed likely that they were altering the membrane fatty acid composition of strain MOR401. Accordingly, the fatty acid compositions of cells grown in the absence and presence of 1 mM concentrations of the BCCAs at 37°C were determined. The gas liquid chromatograph traces are shown in Fig. 1 (Supplemental) and the fatty acid compositions are shown in Table 2. Strain MOR401 has a significantly different fatty acid composition from its parent strain 10403S (Table 2). Ninety eight per cent of the fatty acids were BCFAs in the parent strain, with the major fatty acids being anteiso C15:0 and C17:0 and iso C15:0. In contrast BCFAs only made up 33% of the total in strain MOR401 and the major fatty acids were C16:0, C14:0 and iso C16:0 (Table 2 and Fig 1a supplemental). However, when strain MOR401 was grown in the presence of 2-MB two major peaks appeared in the gas chromatograph trace with retention times of 2.44 and 3.08 minutes (Fig. 1b supplemental) that were identified as anteiso C15:0 (52.3%) and anteiso C17:0 (40.8%).The results are in perfect

accord with previous studies on strain *cld-2* [2, 5, and 12]. Growth in the presence of 2-MP led to two novel major peaks in the chromatograph with retention times of 2.61 and 3.24 min (Fig. 1c supplemental), respectively, constituting 64.5 and 12.3% of the total fatty acids. In fatty acid biosynthesis two carbon atoms are added at a time to a precursor CoA molecule until the fatty acid chain reaches the required length for incorporation into the membrane [18]. In this case the precursor molecule is postulated to be 2-MP-CoA and the two fatty acids are postulated to be 12-methylpentadecanoic acid (C16) and 14-methylheptadecanoic acid (C18). Similarly, the presence of 1 mM 2-EB in the medium resulted in two major peaks of retention times 2.75 and 3.38 min, respectively constituting 50.4 and 31.6% of the total fatty acids (Fig. 1d supplemental). These fatty acids are postulated to be 12-ethyltetradecanoic acid (C16) and 14-ethylhexadecanoic acid (C18) respectively. Very similar results were found when the experiments were performed with strain *cld-2* (unpublished observations). Mass spectral analysis (Fig. 2a and b) revealed a spectrum corresponding to an ethyl branch on the 12^{th} carbon of a 14 carbon chain (peak one) and an ethyl branch on the 14^{th} carbon of a 16 carbon chain (peak two) of these two major peaks in the gas chromatogram. Clearly 2-MP and 2-EB led to the production of novel fatty acids that under normal circumstances would not normally be found in a bacterial membrane. However, their physical structures apparently endow the membrane with biophysical properties that result in stimulation of the growth of the BCFA-deficient mutant at both temperatures.

In order to achieve membrane homeoviscosity at low temperatures *L. monocytogenes* increases the proportion of fatty acid anteiso C15:0 in its lipids [2, 5, 8], by fatty acid chain shortening and branching switching. It was therefore of interest to observe whether any changes occurred in the proportions of the unnatural even-numbered fatty acids in response to growth at low temperatures. When grown in the presence of 2-MP at 10°C, 10-methyltridecanoic acid

increased from 1.04 to 14.4%, 14-methylheptadecanoic acid decreased from 12.3 to 0.5% in strain MOR401 and 12-methylpentadecanoic acid increased to 66% from 64% (Table 3) compared to cells grown at 37ºC (Table 2). Similarly, when grown at 10°C in the presence of 2-EB, 12-ethyltetradecanoic acid increased to 65.9% from 50%, and 14-ethylhexadecanoic acid decreased to 14.2% from 31.6% (Table 3) compared to cells grown at 37ºC (Table 2). Thus, even with the novel BCFAs the cells can execute successful homeoviscous adaptation at low temperature due to fatty acid shortening.

3.3.Incorporation of unnatural even-numbered BCFAs results in increased membrane fluidity

It was expected that given the incorporation of large amounts of the novel fatty acids and the enhancement of the growth of the mutant the membrane fluidity of the cells grown in the presence of 2-MP and 2-EB would be enhanced compared to growth in unsupplemented BHI medium. When strain MOR401 was grown in unsupplemented BHI medium at 37ºC it exhibited a polarization value of 0.24, which was much higher than that observed in the parent strain (0.17) under the same conditions, and indicates a less fluid membrane (Fig. 3). This directly correlates with the deficiency of odd-numbered anteiso fatty acids along with a high proportion of SCFAs and even iso fatty acids in the mutant. When the medium was supplemented with the different BCFA precursors, there was a considerable decrease in the anisotropy values confirming an increase in the fluidity of the membrane (Fig. 3). Inclusion of 2-MB restored the fluidity close to the wild-type, as did 2-EB and 2-MP (Fig. 3).

3.4. BCCA supplementation helps restore normal cell division of the BCFA-deficient mutant

Light microscopic analysis of the bacterial cells showed that when grown in unsupplemented BHI broth, strain MOR401 appeared to be unusually long (almost 3µm) with irregular division

sites (identified by arrows in Fig. 4), in contrast to the wild type cells which are short rods. It seems that the BCFA deficiency and decreased membrane fluidity interfere with proper division of the mutant. Supplementation with 2-MB, which reinstates BCFA content and fluidity of the membrane, restored normal division of the cells. 2-EB also showed similar effects on cell division which indicates the novel BCFAs generated from synthetic substrates can also provide the appropriate biophysical properties to the membrane for normal cell division.

3.5 A 2-position branch in the exogenous BCCA precursors effectively rescued the growth of the mutant

A range of BCCAs, with variations in chain lengths and branching patterns, were studied for their ability to stimulate the growth of the strain MOR401 in order to study the fundamental structural requirements for functioning as an efficient fatty acid precursor. The mutant was grown in the presence of each of these precursors at 37ºC and the growth kinetics were determined (Fig. 5) Like butyrate none of the corresponding straight-chain substrates - pentanoate (C5), hexanoate (C6) or heptanoate (C7) had any influence on growth of the mutant (data not shown). Among the various BCCA precursors studied of varying chain lengths, the position of branching and nature of branching, a methyl, dimethyl or ethyl branch at the 2 position were found to be effective in stimulating the growth of the mutant at 37ºC (Fig. 5). Trimethyl acetate and 2,2-dimethylbutyric acid were efficient in rescuing growth at 37ºC, but to a lesser extent than 2-MB. Trimethyl acetate supplementation yielded two primary novel peaks of retention times 2.26 and 2.89 which are postulated to be 12-dimethyltridecanoic acid and 14-dimethylpentadecanoic acid respectively, each constituting about 20% of the total membrane fatty acid composition (Table 2). 2,2-dimethylbutyrate supplementation also had a similar outcome with the membrane having a total of 85% BCFA of which 12,12-dimethyltetradecanoic acid (retention time 2.57 min) and 14,14-dimethylhexadecanoic acid(retention time 3.21 min) were postulated to be the novel fatty acids.

However these precursors were unable to support the growth of the mutant at low temperatures. The fluidity of the membrane was moderately increased by the dimethyl branched precursors (Fig. 3).

Although we observed that a branch at the second position was crucial for the precursors to rescue the growth of the mutant, the length of the carbon chain plays a role too. Among the 2 carbon-branched BCCAs we tested, IB, 2-MB, 2-MP (chain lengths of 3, 4 and 5 respectively) were highly efficient in stimulating the growth of the BCFA mutant. However, the efficacy decreased drastically when 2-methylhexanoate was used as a substrate, which has a chain length of 6 carbons. This decreased efficiency may be because of inefficient conversion to the corresponding CoA derivative by the bypass pathway enzymes, or inefficient utilization of the CoA precursors by FabH.

4. Discussion

a. Fatty acid biosynthesis in the BCFA-deficient mutant

Strain MOR401 contains a Tn*917* insertion in the *lpd* gene of the *bkd* operon and thus has a nonfunctional branched-chain keto acid dehydrogenase enzyme complex. This leads to a strikingly different fatty acid composition than the parent strain, 10403S, in which even numbered SCFAs make up about 65% of the total fatty acids and iso-even numbered fatty acids about 25% in the mutant. The iso-even numbered fatty acids are biosynthesized from isobutyryl-CoA. Under normal circumstances this fatty acid primer is produced from the branched-chain amino acid valine via branched-chain amino acid transaminase followed by branched-chain keto acid dehydrogenase activities (Fig. 6). This route is not operative in strain MOR401 [5]. Potential alternative routes to

isobutyryl-CoA include from valine via valine dehydrogenase, and by isomerization of butyryl-CoA [2].

FabH carries out the first condensation reaction in fatty acid biosynthesis [18]. Butyryl-CoA is likely to be the precursor for the SCFAs found in high amounts in MOR401 because *L. monocytogenes* FabH has very low activity with acetyl-CoA, the only other likely possible precursor of even numbered SCFAs [19]. However, there does not appear to be any information on how butyryl-CoA might be formed endogenously in *L. monocytogenes*. It is not known whether butyrate is present in BHI medium to act as a source of butyryl-CoA. However, when strain *cld-2* (from which MOR401 is derived) is grown in defined medium without butyrate the strain's fatty acid composition is also characterized by a high proportion of even numbered SCFAs [2]. This would also argue against longer chain fatty acids being incorporated into the phospholipids of the organism from the growth medium, such as happens in *S. aureus* [20]. A possible route to butyryl-CoA is via the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, a reaction catalyzed by the enzyme thiolase followed by a series of enzymatic reactions studied extensively in butyrate-producing bacteria such as species of *Clostridium* [21]. When a genome-wide search for these enzymes or their homologs was carried out throughout the *Listeria* genus including *L. monocytogenes* strains 10403S and EGDe, they all seem to be present (data not shown), although not well characterized. Thus it seems feasible that in absence of pathways producing BCFAs in the membrane, butyryl-CoA production and its corresponding elongation leads to SCFAs dominating the membrane profile of the BCFA-deficient mutant.

When MOR401 (or *cld-2*) is supplied with 2-MB in the medium it causes a dramatic switch in fatty acid composition such that anteiso C15:0 and C17:0 become almost 90% of the total fatty acid composition (Table 2) [2, 5]. This clearly results in a membrane with much more ideal

biophysical properties for the organism, enabling it to grow efficiently even at low temperatures. Thus there appears to be a bypass to allow the organism to utilize an exogenous supply of short BCCA precursors yielding acyl-CoA primers ready to enter FASII elongation pathway. 2-MBCoA is the preferred *L. monocytogenes* FabH substrate with the highest activity among various precursors tested [19]. Although the pathway to 2-MBCoA from an exogenous source has not been ascertained yet, the *bkd* operon contains two genes upstream of the *bkd* cluster, *buk* and *ptb,* encoding butyrate kinase (Buk) and phosphotransbutyrylase (Ptb) respectively [5], which could function to produce 2-MBCoA after 2-MB crosses the *L. monocytogenes* cytoplasmic membrane as depicted in Fig 6.

Supply of 2-MP and 2-EB in the growth medium also results in very high proportions of fatty acids in the membrane derived from them, not observed under normal circumstances. Clearly, a pathway must exist to produce 2-MPCoA and 2-EBCoA and these C6 primer molecules are then used efficiently by FabH for elongation and incorporation. Buk and Ptb, which we hypothesize form 2-MBCoA from 2-MB, are also likely to catalyze the formation of the CoA derivatives from 2-MP and 2-EB suggesting a relatively wide substrate specificity of the enzymes. It would be interesting to characterize the kinetic parameters of FabH with these substrates, as well as those of the enzymes in the pathway leading to production of 2-MPCoA and 2-EBCoA. Our unpublished observations show that the C6 BCCA substrates can be efficiently utilized by Ptb.

b. Membrane fluidity and physiological properties of 2-MP- and 2-EB-grown BCFA-deficient mutant

Both 2-MP and 2-EB stimulated growth of MOR401 at 37ºC and restored growth of the strain at 10ºC. The main fatty acids generated from these precursors, 12-methylpentadecanoic

acid and 12-ethyltetradecanoic acid respectively, although unnatural provide the membrane with biophysical properties that restore its functional efficiency. Strain MOR401 is impaired in cell division producing short chains of unusually long and irregularly dividing cells. Inclusion of 2-MB, 2-MP, or 2-EB in the medium corrects this and restores the normal cellular arrangement of the strain. This suggests that low membrane fluidity somehow interferes with the complex process of cell division carried out by the divisome [22], and when the ideal fluidity is restored through sufficient BCFA production in the membrane the cell regains its normal physiology and can divide successfully. This proves that as long as the fatty acid molecule can provide the correct biophysical properties it does not necessarily have to be a natural cellular product. A suitable synthetic precursor can be very well utilized by the bacterial system to restore its ideal membrane parameters. Mercier *et al*. [23] have demonstrated a crucial role for BCFAs and their associated membrane fluidity in membrane scission in "L-form" *B. subtilis* cells.

The impact of the natural anteiso fatty acids having a methyl branch at the antepenultimate position such as anteiso C15:0 on the fluidity of the cytoplasmic membranes is attributed to the larger cross-sectional area they occupy than the corresponding SCFAs. This increases the area per lipid, and disrupts close packing of the fatty acyl chains and chain order along with reducing thickness of the lipid bilayer [15, 24]. A recent study on the effects of methyl branched fatty acids on the structural properties of the lipid bilayer using a 1,2-dipalmitoyl-sn glycerol-3-phosphocholine lipid bilayer, showed that the position of the methyl branch on the fatty acyl chain directly influences the membrane fluidity, the fluidizing ability of a mid-chain branch being greater than a terminal one [24]. The branching of 2-MP and 2-EB-derived fatty acids also no doubt occupy a large cross-sectional area similar to the naturally occurring BCFAs, thereby also imparting significant fluidity to the membrane.

c. Psychrotolerance of L. monocytogenes

In order to survive in the cold, psychrophiles must have enzymes that perform effectively at low temperatures. Indeed, cold environments reduce enzyme reaction rates and increase membrane viscosity. Cold-adapted organisms cope with these conditions by increasing enzyme turnover or improved catalytic efficiency at low temperatures compared to homologous enzymes in mesophiles. A commonly accepted hypothesis for cold adaptation is that psychrophilic enzymes have an increased flexibility of their structure to compensate for the "freezing effect" at cold temperatures [25]. Such changes are not without cost as this increased flexibility is likely responsible for the lower protein stability generally associated with cold-adapted enzymes, especially at higher temperatures.

L. monocytogenes is not psychrophilic, but rather psychrotolerant, which sets up a paradoxical survival problem for this organism. It must be able to thrive at 37°C and thus cannot afford thermally unstable enzymes, yet it maintains the ability to grow at refrigeration temperatures, which presumably requires increased enzymatic flexibility. Previously, we revealed that *L. monocytogenes* was uniquely equipped to handle just such a paradox, in that even during exponential growth at 37°C, anteiso-C15:0 accounted for 48% of the total fatty acids [2]. Although other Gram positive bacteria incorporate BCFAs into their membranes, their levels of anteiso-C15:0 are consistently ~30%, comparable to the cold-sensitive *L. monocytogenes* mutants, *cld-1* and *cld-2* and MOR401 [2, 5]. Comparative analysis of the crystal structures of several bacterial FabH enzymes suggests a molecular basis for their substrate specificity [26]. Steric interactions between conserved physically close phenylalanines (distant in primary structure, e.g., between F^{305} and F^{208} in *L. monocytogenes*) cause a narrowing of the FabH active site in Gram-positive bacteria. The perturbing residue (i.e. F^{208}) is not well conserved in Gram-negative bacteria (e.g. V^{216} in *E.*

coli), which then allows the active site phenylalanine residue to swing away from the active site and open the substrate cavity [26]. Substrate specificity of FabH is the determining factor in the biosynthesis of BCFAs by type II fatty acid synthases [27, 28]. Accordingly, FabH enzymes from organisms that produce BCFAs exhibit broader substrate specificity than FabH homologues from organisms which produce SCFAs [27]. It is this broader substrate utilization that permits the phenomenon of homeoviscous adaptation to low temperatures by fatty acid branch switching and chain length shortening which occurs in wild-type *L. monocytogenes* and BCFA-deficient mutants in growth medium supplemented with 2-MB [2, 5, 8].

An increase in the proportion of the shorter fatty acids derived from 2-MP and 2-EB accompanied by a decrease in the proportion of the longer ones was also observed in adaptation to low temperature. Thus *L.monocytogenes* can also carry out homeovisocus adaptation with the novel fatty acids derived from these unique precursors.

d. Structural requirements for a precursor of fatty acid biosynthesis

A variety of short BCCAs were evaluated for their ability to act as fatty acid primers. Along with the various ones with a methyl branch at position 2 on the acyl chain, trimethyl acetate and 2,2-dimethylbutyric acid also rescued the growth of the BCFA- deficient mutant to significant extents at 37ºC but not at 10ºC. Precursors having a branch at the third position such as 3-MP or isovalerate failed to support growth of MOR401 or *cld-2* [5]. This indicates that a branch at the 2-position is an important structural parameter of the precursors for fulfillment of this function. Both Kaneda [13] and Willecke and Pardee [15] found that branching at the 2-position was important for BCCAs to act as fatty acid primers in *B.subtilis*. However, they did not include studies of the efficiency of these precursors at low temperatures. Although it is possible that the poor ability of

3-branched BCCAs to act as primers may lie in a low efficiency of forming the CoA derivatives, we feel that it is more likely that the defect lies in the substrate preferences of FabH.

The fatty acid profile of the membrane does show incorporation of novel BCFAs when the mutant is grown in presence of trimethyl acetate or 2,2-dimethylbutyrate. The novel fatty acids generated from trimethyl acetate and 2,2-dimethylbutyrate totaled 41.3% and 29.5% respectively compared to 82%, 76.8% and 93% from 2-EB, 2-MP and 2-MB respectively, all of which had a much larger impact on the fatty acid composition of MOR401 grown in unsupplemented medium. The major impact of trimethyl acetate and 2,2-dimethylbutyrate was to diminish the even-SCFA content, whereas the iso-odd BCFAs actually increased. Assuming these two BCCAs permeate the membrane efficiently we suspect the two groups at the 2-position confer a bulkier structure that renders these compounds to be poor substrates for either the enzymes in the pathway forming their CoA derivatives, or their utilization by FabH (or both). These BCCA precursors stimulate growth at 37ºC, and presumably increase the fluidity of the membrane sufficiently, even though we did not detect this in our anisotropy measurements, which may miss subtle, but important fluidity changes.. We propose that conversion of trimethyl acetate and 2,2-dimethylbutyrate to CoA derivatives and/or their utilization by FabH are too inefficient at 10ºC for stimulation of growth of MOR401.

Our observations raise the question of how the preference for branched-chain precursors can be reconciled with a sterically narrowed FabH substrate access site as identified by Gajiwala [26]. Also how does the structure of the active site account for a selective difference between 2- and 3-branched precursors? The definitive answers await a *L. monocytogenes* FabH crystal structure with a bound branched-chain precursor. However, one hypothesis is that the impinging phenylalanine may allow stabilizing Van der Waals interactions with an alkyl group on the 2-

position of the CoA precursor. The increased contact likely stabilizes the acyl-CoA precursor for efficient catalysis to occur. In the case of a branch in the 3-position, the same stabilizing contact would leave a two-carbon overhang which would likely be a similar poor substrate for *L. monocytogenes* FabH as acetyl-CoA [19].

e. Concluding remarks

Sun *et al*. [11, 12] reported 2-MB supplementation in the BCFA-deficient mutant plays a key role in expression of virulence properties such as listeriolysin O and survival against CAMPs, and peptidoglycan hydrolases and survival in an *in vivo* model. Since the novel fatty acids produced from 2-MP and 2-EB also lead to similar membrane biophysical properties in the mutant as when anteiso C15:0 and C17:0 are produced from 2-MB,, we can predict that they may have similar effects on the physiology and pathogenicity of the pathogen. In support of this 2-MB, 2-MP and 2-EB all corrected the defective cell division of the mutant. In terms of modulating the fatty acid composition of wild-type *L. monocytogenes* in order to inhibit growth in food, or in vivo, increasing the content of SCFAs from butyrate appears to be the most effective way. The current studies illustrate an interesting picture as to how the putative fatty acid metabolic enzymes in *Listeria* adapt themselves efficiently to form novel products from synthetic precursors by non-native promiscuity to support the membrane integrity for proper survival. Future studies will investigate the functional properties of the membranes containing large amounts of unnatural fatty acids.

Acknowledgments:

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no role in study design, collection, analysis and interpretation of data, writing of this manuscript or the decision to submit it for publication. We would like to thank Dr Charitha Galva, at Illinois State University, for her help with the images for our manuscript. We would also like to acknowledge Lily Fernandez-Flores, a MS student in our lab, for her preliminary studies with the BCFA-deficient mutant.

Table 1. Various branched-chain carboxylic acids with diverse branching pattern and chain lengths

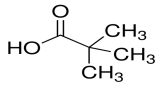
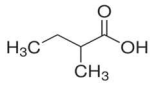
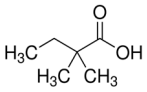
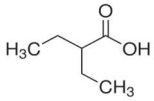
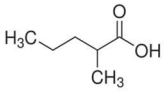
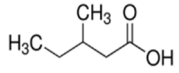
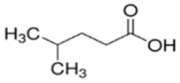
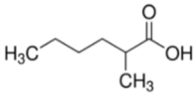
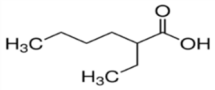
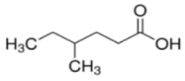
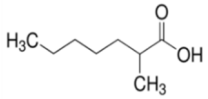
Fatty acid precursors	Chemical structure	Branch	Chain length of hydrocarbon backbone
Trimethyl acetate (C5)		2,2-dimethyl	3C
2-methylbutyrate (C5)		2 methyl	4C
2,2-dimethylbutyrate (C6)		2,2-dimethyl	4C
2-ethylbutyrate (C6)		2 ethyl	4C
2-methylpentanoate (C6)		2 methyl	5C
3-methylpentanoate (C6)		3 methyl	5C
4-methylpentanoate (C6)		4 methyl	5C
2-methylhexanoate (C7)		2 methyl	6C
2-ethylhexanoate (C8)		2 ethyl	6C
4-methylhexanoate (C7)		4 methyl	6C
2-methylheptanoate (C8)		2 methyl	7C

Table 2. The membrane fatty acid profile of *L. monocytogenes* parent strain 10403S and the BCFA-deficient mutant MOR401 grown in BHI at 37°C with or without BCCA precursors

% (wt/wt) of total fatty acids

Strain, growth conditions	*Anteiso odd*			*Iso odd*	*Iso even*	*Straight even*	*Novel fatty acids*			*BCFA*	*SCFA*
	C15:0	C17:0	SUM								
10403S	47.77±0.59	34.76±0.4	82.5	11.7	3.82	1.94	ND	ND	ND	98.07	1.94
MOR401	5.4±1.9	2±1.8	7.4	1.6	24.1	57.2	ND	ND	ND	33.2	66.0
MOR401+2MB	52.3±1.5	40±0.7	93.1	ND	0.74	6.2	ND	ND	ND	93.8	6.2
MOR401+2EB	0.6±0.01	1.8±0.3	2.3	ND	2.39	12.4	ND	12-ethyltetradecanoic acid 50.4±2.7	14-ethylhexadecanoic acid 31.6±0.6	86.8	13.3
MOR401+2MP	1.4±0.5	2.4±0.7	3.8	ND	10.5	5.4	10-methyltridecanoic acid 1.04±0.08	12-methylpentadecanoic acid 64.5±0.6	14-methylheptadecanoic acid 12.3±2.6	92.4	6.8
MOR401+trimethyl acetate	7.6±1.3	10.2±0.1	17.8	ND	28.1	11.2	ND	12-dimethyltridecanoic acid 21.1±1.4	14-dimethylpentadecanoic acid 20.2±1.4	87.2	11.2
MOR401+2,2-dimethylbutyrate	9.5±1.5	12.02±0.01	21.5	ND	35.3	10.8	ND	12,12-dimethyltetradecanoic acid 21.5±1.6	14,14-dimethylhexadecanoic acid 8±0.6	86.3	13.7

All supplements were used at 1 mM.

The percentages of respective fatty acids are means from three independent experiments with standard deviations

ND- Not detected

The minor fatty acids (<1%) are not reported. This includes minor percentages of odd numbered SCFAs in some cases.

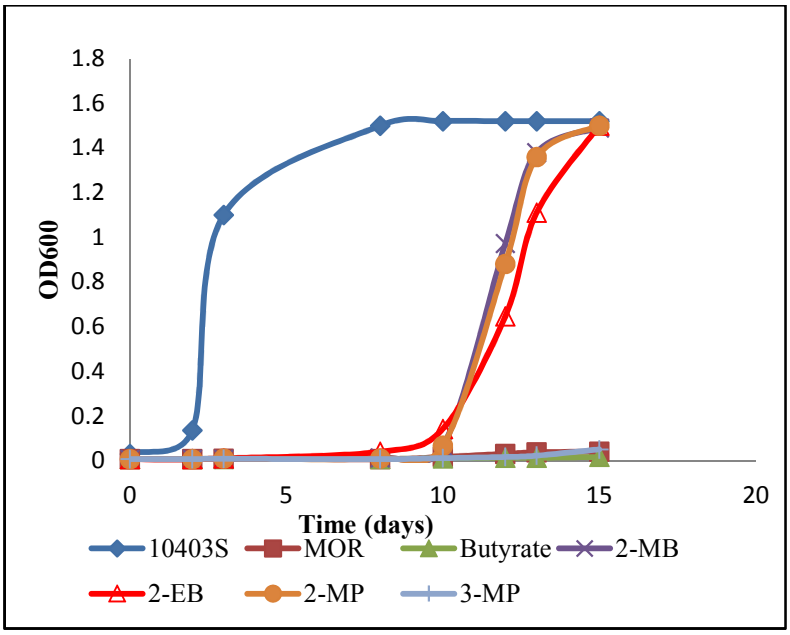
Table 3. The membrane fatty acid profile of *L. monocytogenes* parent strain 10403S and the BCFA-deficient mutant MOR401 supplemented with BCCA precursors grown in BHI at 10°C

% (wt/wt) of total fatty acids

Strain, growth conditions	*Anteiso odd*			*Iso odd*	*Iso even*	*Novel fatty acids*			*BCFA*	*SCFA*
	C15:0	C17:0	SUM							
10403S	69.9±1.5	7.6±0.8	78.6	15.2	4.9	ND	ND	ND	98.8	0.6
MOR401 +2MB	77.9±1.4	15.8±0.5	94.7	ND	0.1	ND	ND	ND	95.3	3.6
MOR401 + 2EB	1.4±0.21	1.5±0.7	2.9	ND	4.3	ND	12-ethyltetradecanoic acid 65.9±5.5	14-ethylhexadecanoic acid 14.2±2.6	87.7	10.8
MOR401 +2MP	1.7±0.18	0.6±0.8	2.3	ND	11.8	10-methyltridecanoic acid 14.4±5	12-methylpentadecanoic acid 65.9±0.42	14-methylheptadecanoic acid 0.5±0.63	94.2	4.5

All supplements were used at 1 mM.
The percentages of respective fatty acids are means from three independent experiments with standard deviations
ND- Not detected
The minor fatty acids (<1%) are not reported.

a



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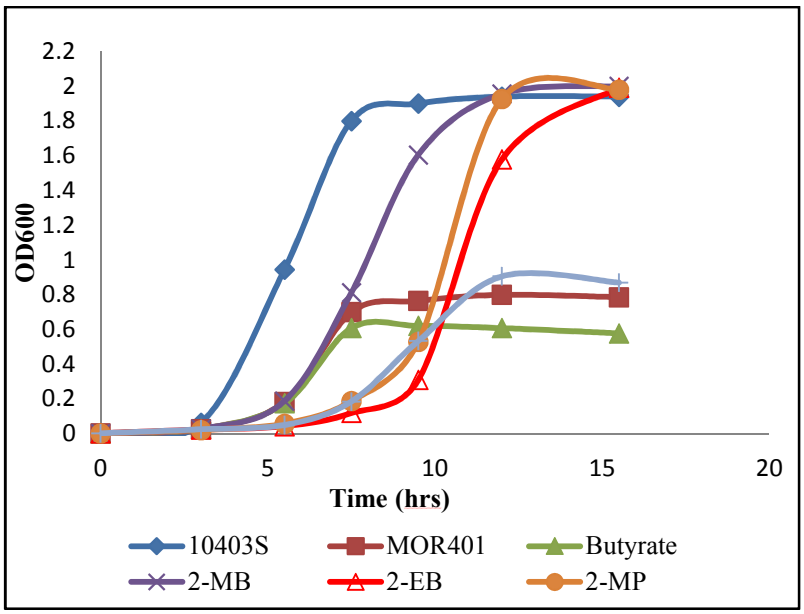
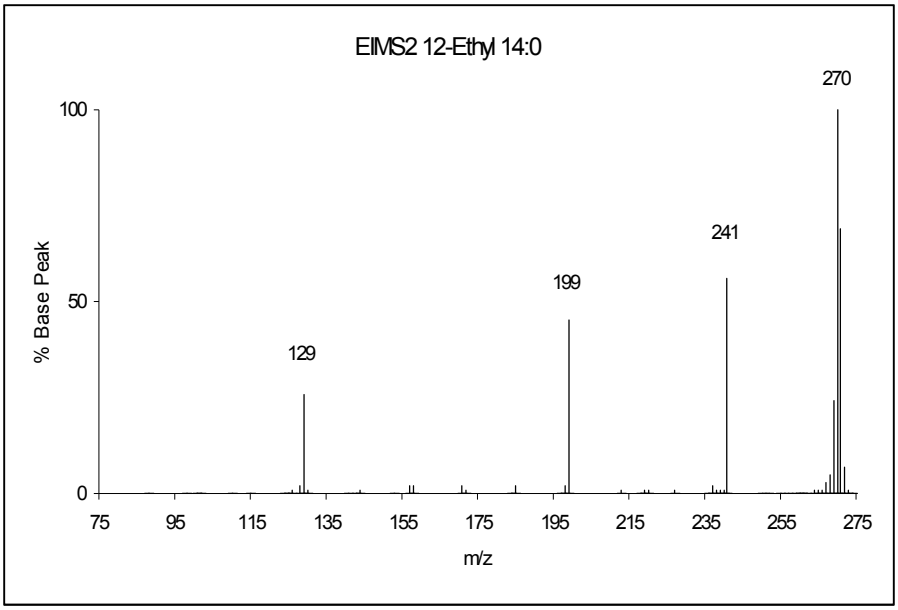


Fig.1. Influence of the BCCA precursors on the growth of the BCFA-deficient mutant MOR401 (a) at 37ºC and (b) at 10ºC Symbols: (♦) parent strain 10403S; all other symbols are strain MOR401 with no supplementation (■) or supplemented with butyrate (▲) 2-MB (x) 2-EB (Δ) 2-MP (●) 3-MP (+) Representative figures from triplicate experiment sets are shown.

a



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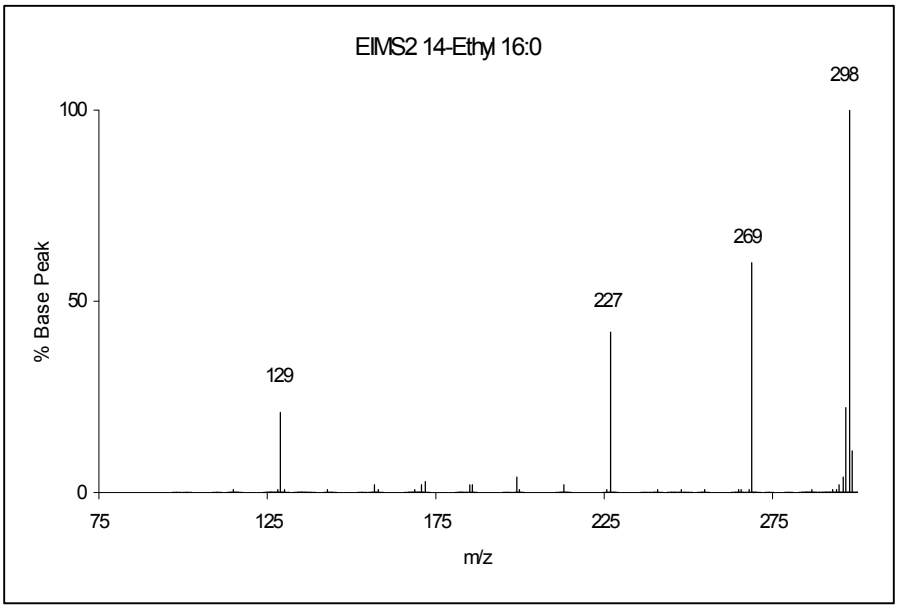

Fig. 2. Mass spectral analysis of membrane fatty acids of the BCFA-deficient mutant strain MOR401 when grown in presence of 2-EB

Two novel fatty acid methyl ester peaks with retention times (a) 2.75 min and (b) 3.38 min from the GLC analysis were subjected to mass spectral analysis. The peaks yielded spectra that correspond to (a) an ethyl branch on the 12 carbon of a 14 carbon chain, and (b) on the 14th carbon on a 16 carbon chain.

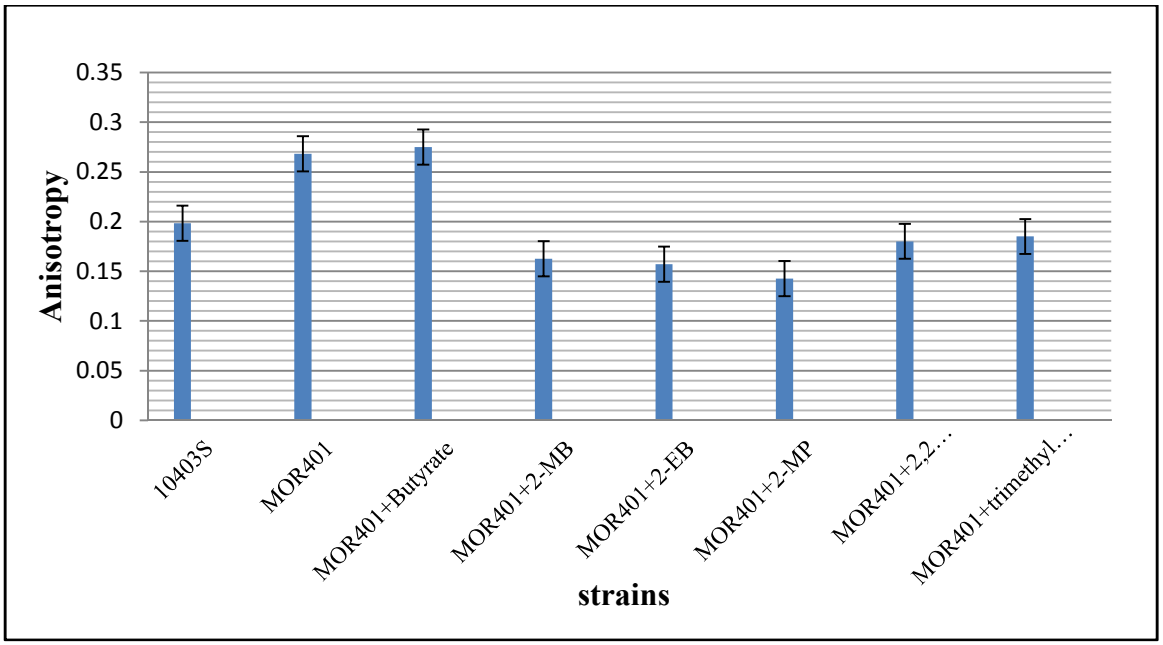

Fig. 3. Influence of the short chain carboxylic acid precursors on the membrane fluidity of the BCFA-deficient mutant strain MOR401 at 37ºC

The strains were grown in medium supplemented with the indicated fatty acid precursors and membrane anisotropy was measured by fluorescence polarization.

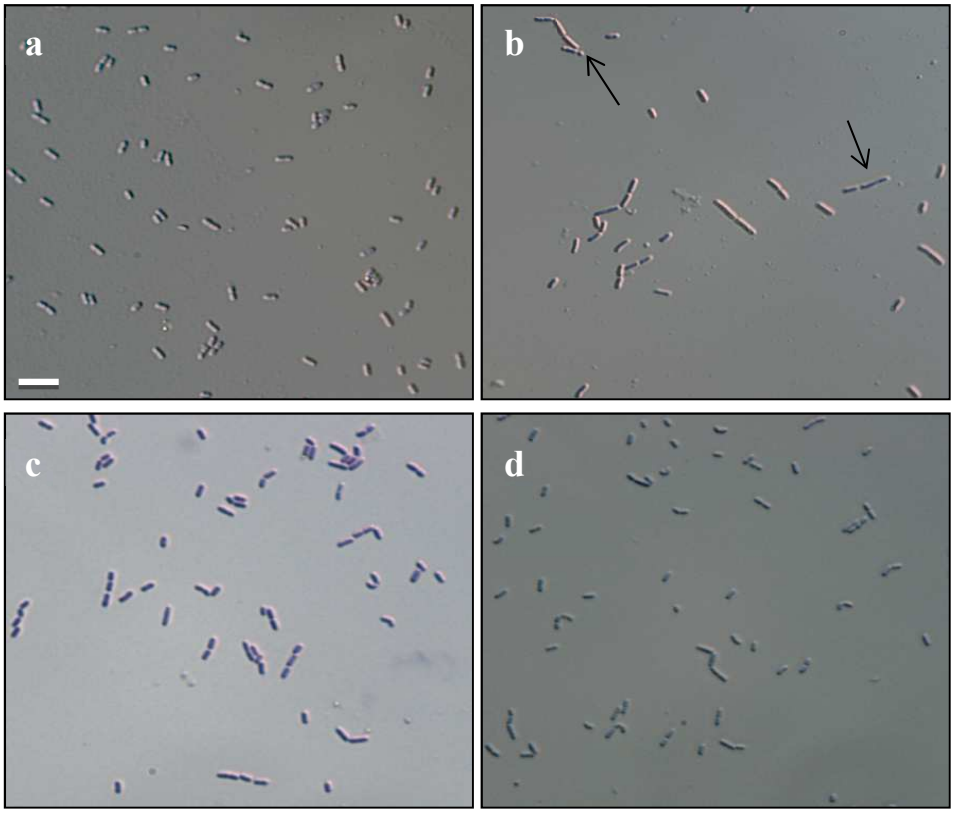

Fig.4. Light microscopic analysis of the BCFA-deficient mutant MOR401 under the influence of different fatty acid precursors

Compared to the wild type *Listeria monocyogenes* 10403S cells (a), the BCFA-deficient mutant grown in unsupplemented BHI (b) is unusually long and exhibits irregular division sites (pointed out by arrows) Precursors 2-MB (c) and 2-EB (d) restored the cell dimensions and normal cell division. **Scale bar**= 4 μm

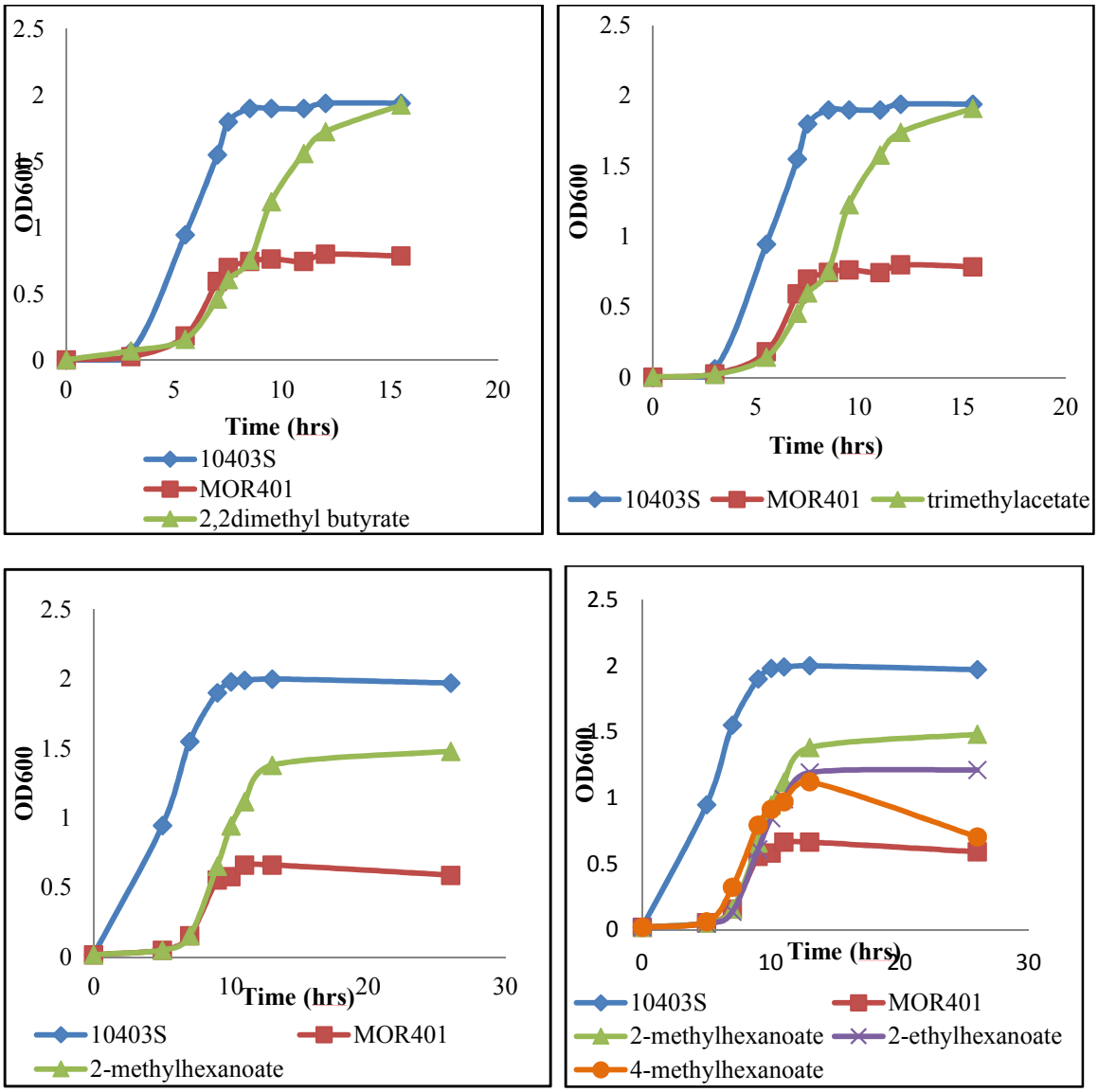

Fig.5. Influence of various short BCCA precursors on the growth of the BCFA-deficient mutant strain MOR401 at 37ºC

Representative figures from triplicate experiment sets are shown. (a) ♦ parent strain 10403S; ■ MOR401 with no supplementation; ▲ MOR401 with 2,2-dimethylbutyrate (b)♦ parent strain 10403S; ■ MOR401 with no supplementation; ▲ MOR401 with trimethylacetate (c)♦ parent strain 10403S; ■ MOR401 with no supplementation; ▲ MOR401 with 2-methylhexanoate (d) ♦ parent strain 10403S; ■ MOR401 with no supplementation; ▲ MOR401with 2-methylhexanoate; x MOR401with 2-ethylhexanoate; ● MOR401with 4-methylhexanoate

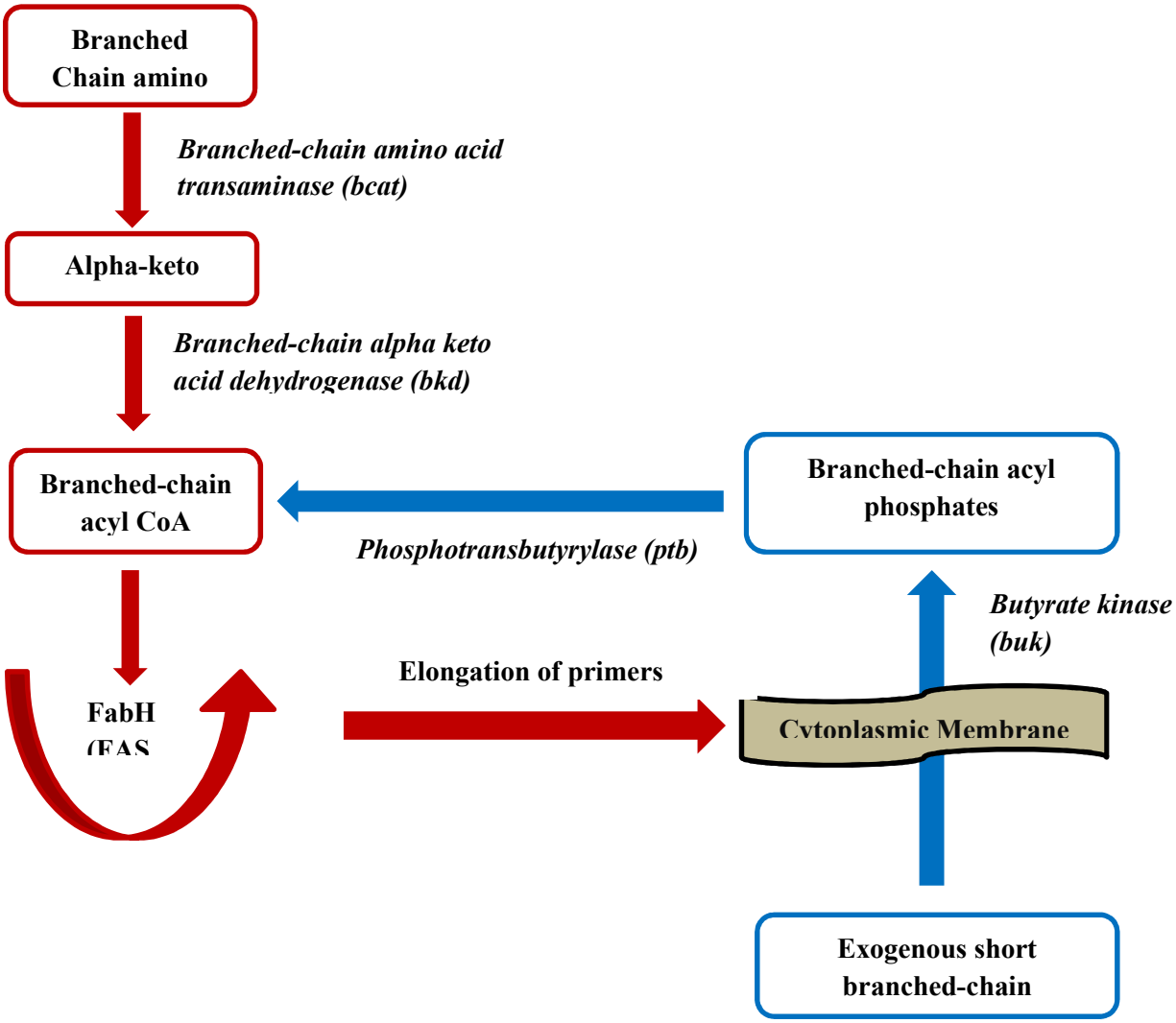

Fig.6. Endogenous pathway for membrane BCFA production and the putative pathway for exogenous BCCA utilization in *L. monocytogenes*

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CHAPTER II

GROWTH-ENVIRONMENT DEPENDENT MODULATION OF *STAPHYLOCOCCUS AUREUS* BRANCHED-CHAIN TO STRAIGHT-CHAIN FATTY ACID RATIO AND INCORPORATION OF UNSATURATED FATTY ACIDS

Abstract

The fatty acid composition of membrane glycerolipids is a major determinant of *Staphylococcus aureus* membrane biophysical properties that impacts key factors in cell physiology including susceptibility to membrane active antimicrobials, pathogenesis, and response to environmental stress. The fatty acids of *S. aureus* are considered to be a mixture of branched-chain fatty acids (BCFAs), which increase membrane fluidity, and straight-chain fatty acids (SCFAs) that decrease it. The balance of BCFAs and SCFAs in USA300 strain JE2 and strain SH1000 was affected considerably by differences in the conventional laboratory medium in which the strains were grown with media such as Mueller-Hinton broth and Luria broth resulting in high BCFAs and low SCFAs, whereas growth in Tryptic Soy Broth and Brain-Heart Infusion broth led to reduction in BCFAs and an increase in SCFAs. Straight-chain unsaturated fatty acids (SCUFAs) were not detected. However, when *S. aureus* was grown *ex vivo* in serum, the fatty acid composition was radically different with SCUFAs, which increase membrane fluidity, making up a substantial proportion of the total (<25%) with SCFAs (>37%) and BCFAs

(>36%) making up the rest. Staphyloxanthin, an additional major membrane lipid component unique to *S. aureus* tended to be greater in content in cells with high BCFAs or SCUFAs. Cells with high staphyloxanthin content had a lower membrane fluidity that was attributed to increased production of staphyloxanthin. *S. aureus* saves energy and carbon by utilizing host fatty acids for part of its total fatty acids when growing in serum, which may impact biophysical properties and pathogenesis given the role of SCUFAs in virulence. The nutritional environment in which *S. aureus* is grown *in vitro* or *in vivo* in an infection is likely to be a major determinant of membrane fatty acid composition.

Introduction

Staphylococcus aureus is a worldwide significant pathogen in the hospital and the community. Antibiotic resistance has developed in waves such that we now have methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) [2,3]. Given the threat of multiply antibiotic-resistant *S. aureus*, various aspects of staphylococcal biology including pathogenicity, antibiotic resistance, and physiology are currently being investigated intensively, in part to support the search for novel anti-staphylococcal agents.

The bacterial cytoplasmic membrane forms an essential barrier to the cell and is composed of a glycerolipid bilayer with associated protein molecules, and is a critical determinant of cell physiology. The biophysical properties of the membrane are to a large extent determined by the fatty acyl residues of membrane phospholipids and glycolipids [4,5]. The lipid acyl chains influence membrane viscosity/fluidity, and impact the ability of bacteria to adapt to changing environments, the passive permeability of hydrophobic molecules, active transport, and the

function of membrane-associated proteins [4–6]. Additionally, membrane fatty acid composition has a major influence on bacterial pathogenesis, critical virulence factor expression [7], and broader aspects of bacterial physiology [8].

S. aureus membrane fatty acids are generally considered to be a mixture of branched-chain fatty acids (BCFAs) and straight-chain fatty acids (SCFAs) [9–11], and for a comprehensive review of earlier literature see [12]. In *S. aureus* the major BCFAs are odd-numbered iso and anteiso fatty acids with one methyl group at the penultimate and antepenultimate positions of the fatty acid chains, respectively (Fig. 1). BCFAs have lower melting points than equivalent SCFAs and cause model phospholipids to have lower phase transition temperatures [13], and disrupt the close packing of fatty acyl chains [14,15].

Fatty acids are major components of the *S. aureus* phospholipids, which are phosphatidyl glycerol, cardiolipin and lysysl-phosphatidyl glycerol [16]. BCFAs are biosynthesized from the branched-chain amino acids, isoleucine (anteiso odd-numbered fatty acids), leucine (iso odd-numbered fatty acids), and valine (iso even-numbered fatty acids) via branched-chain aminotransferase and branched-chain α- keto acid dehydrogenase [13]. The branched-chain acyl CoA precursors thus formed are used for the biosynthesis of fatty acids by the dissociated bacterial fatty acid synthesis system (FASII) [5,17]. Phosphatidic acid is a key intermediate in the biosynthesis of the *S. aureus* phospholipids [5]. Our current knowledge of the pathway of phospholipid biosynthesis and the incorporation of exogenous and endogenous fatty acids is summarized in Fig. 2 [18]. Phosphatidic acid, the universal precursor of phospholipids, is synthesized by the stepwise acylation of *sn*-glycerol-3-phosphate first by PlsY that transfers a fatty acid to the 1-position from acyl phosphate. The 2-position is then acylated by PlsC utilizing acyl-ACP. Acyl-ACP is produced by the FASII pathway and PlsX catalyzes the interconversion

of acyl-ACP and acyl phosphate. When *S. aureus* is grown in medium that results in a high proportion of BCFAs the major phospholipid, phosphatidyl glycerol, has, almost exclusively, anteiso C17:0 at position 1 and anteiso C15:0 at position 2 [17].

The membrane lipid composition of *S. aureus* is further complicated by the presence of staphyloxanthin, a triterpenoid carotenoid with a C30 chain with the chemical name of α-D-glucopyranosyl-1-*O*-(4,4'-diaponeurosporen-4-oate)-6-*O* (12-methyltetradecanoate) [19](Fig. 1). Staphyloxanthin, as a polar carotenoid, is expected to have a significant influence on membrane properties with the expectation that it rigidifies the membrane [20], and Bramkamp and Lopez [21] have suggested that staphyloxanthin is a critical component of lipid rafts in *S. aureus* incorporating the organizing protein flotillin. Staphyloxanthin has drawn considerable attention in recent years as a possible virulence factor by detoxifying reactive oxygen species produced by phagocytic cells [22,23] , and as a potential target for antistaphylococcal chemotherapy [24].

In our laboratory, we are interested in the mechanisms of action of and resistance to novel and existing anti-staphylococcal antimicrobials [25–27]. Because much antibiotic work employs Mueller-Hinton (MH) medium, [28] we had occasion to determine the fatty acid composition of a *S. aureus* strain grown in this medium. The analysis was carried out using the MIDI microbial identification system (Sherlock 4.5 microbial identification system; Microbial ID, Newark, DE, USA), [29]. We were taken aback when the fatty acid profile came back showing a very high percentage (84.1%) of BCFAs, and the organism was not even identified by MIDI as a *S. aureus* strain. In a previous study where we grew *S. aureus* in BHI broth we found that 63.5% of the fatty acids were BCFAs, and 32.4% were SCFAs [10]. This is a much more typically observed balance between BCFAs and SCFAs in previous studies of the fatty acid composition of *S. aureus* [9–12].

A range of different media are used for cultivating *S. aureus* in studies from different laboratories [30]. These are mostly complex media such as Tryptic Soy Broth (TSB), BHI broth, MH broth, Luria-Bertani (LB) broth, and, much more rarely, defined media [11]. Ray *et al*. [30] and Oogai *et al* [31] have pointed out that different media have major, but largely unstudied and ignored, effects on the expression of selected target virulence and regulatory genes. Although seemingly prosaic at first glance, issues of choice of strain and medium are nevertheless critical considerations in staphylococcal research [32]. These authors [32], in their recent protocol publication on the growth and laboratory maintenance of *S. aureus,* have suggested that TSB and BHI media are the media of choice for staphylococcal research. In light of recent literature in various microorganisms, it is becoming evident that environment has a tremendous effect on the physiology of different pathogens; hence cells from *in vivo* growth are significantly different from *in vitro* cultured ones. Such distinctions are likely important for studying antimicrobial susceptibilities, drug resistances and pathogenesis.

We decided to carry out a systematic study of the impact of growth medium on the fatty acid and carotenoid composition of *S. aureus* given the large potential impact of these parameters on membrane biophysical properties and its further ramifications. The BCFA: SCFA ratio was significantly impacted by the laboratory medium used, with media such as MH broth encouraging high proportions of BCFAs. However, strikingly, when cells were grown in serum, an *ex vivo* environment, the fatty acid composition changed radically, with straight-chain unsaturated fatty acids (SCUFAs) (Fig. 1), which were not detected in cells grown in laboratory media, making up a major proportion of the total fatty acids. This extreme plasticity of *S. aureus* membrane lipid composition is undoubtedly important in determining membrane physical structure and thereby the functional properties of the membrane. The alterations in the fatty acid composition as a result

of interactions of the pathogen with the host environment may be a crucial factor in determining its fate in the host. Typically used laboratory media do not result in a *S. aureus* membrane fatty acid composition that closely resembles the likely one of the organism growing *in vivo* in a host.

Materials and Methods

Bacterial Strains and Growth Conditions

The primary *S. aureus* strains studied were strain JE2 derived from strain LAC USA300 [33] and strain SH1000. USA300 strain JE2 is a prominent community-acquired MRSA lineage, which is a leading cause of aggressive cutaneous and systemic infections in the USA [1,34,35]. Strain JE2 has a well-constructed diverse transposon mutant library [33]. *S. aureus* strain SH1000, is an 8325-line strain that has been used extensively for many years in genetic and pathogenesis studies [36]. The laboratory media used were MH broth, TSB, BHI broth and LB from Difco. For growth and fatty acid composition studies cultures of *S. aureus* strains were grown at 37° C in 250 ml Erlenmeyer flasks containing each of the different laboratory media with a flask–to-medium volume ratio of 5:1. Growth was monitored by measuring the OD_{600} at intervals using a Beckman DU-65 spectrophotometer.

Growth of *S. aureus* in Serum

Sterile fetal bovine serum of research grade was purchased from Atlanta Biologics, USA. The aliquoted serum was incubated in a water bath at 56° C for 30 min to heat inactivate the complement system. *S. aureus* cells were grown for 24 hours in 50 ml of serum in a 250 ml flask at 37°C with shaking at 200 rpm.

Analysis of the Membrane Fatty Acid Composition of *S. aureus* Grown in Different Media

The cells grown in the different conventional laboratory media were harvested in mid-exponential phase (OD_{600} 0.6), and after 24 hrs of growth in serum, by centrifugation at 3000 x g

at 4° C for 15 minutes and the pellets were washed three times in cold distilled water. The samples were then sent for fatty acid methyl ester (FAME) analysis whereby the fatty acids in the bacterial cells (30-40 mg wet weight) were saponified, methylated, and extracted. The resulting methyl ester mixtures were then separated using an Agilent 5890 dual-tower gas chromatograph and the fatty acyl chains were analyzed and identified by the MIDI microbial identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc. (Newark, DE) [29]. The percentages of the different fatty acids reported in the tables and figures are the means of the values from three separate batches of cells under each condition. Some minor fatty acids such as odd-numbered SCFAs were not reported.

Extraction and Estimation of Carotenoids

For quantification of the carotenoid pigment in the *S. aureus* cells grown in different media, the warm methanol extraction protocol was followed as described by Davis *et al*. [37]. Cultures of *S. aureus* were harvested at mid-exponential phase and were washed with cold water. The pellets were then extracted with warm (55ºC) methanol for 5 min. The OD_{465} of the supernatant after centrifugation was measured using a Beckman DU 70 spectrophotometer. Determinations were carried out in triplicate. Significant differences between carotenoid content of *S. aureus* grown in different media were determined by analysis of variance (ANOVA) using SAS 9.4 (SAS Institute, NC) with post hoc Tukey's test.

Measurement of the Fluidity of the *S. aureus* Membrane

The fluidities of the cell membrane of the *S. aureus* strains grown in different media were determined by anisotropic measurements using the fluorophore diphenylhexatriene (DPH)

following the protocol described previously [38]. Mid exponential phase cells grown in respective media and serum were harvested and washed with cold sterile PBS (pH 7.5). The pellets were then resuspended in PBS containing 2 μM DPH (Sigma, MO) to an OD_{600} of about 0.3 and incubated at room temperature in the dark for 30 min. Fluorescence polarization emitted by the fluorophore was measured using a PTI Model QM-4 Scanning Spectrofluorometer at an excitation wavelength of 360 nm and emission wavelength of 426 nm. The experiments were performed with three separate fresh batches of cells. Significant differences between mean polarization values of *S. aureus* grown in different media were determined by analysis of variance (ANOVA) using SAS 9.4 (SAS Institute, NC) with post hoc Tukey's test.

Results

The two main strains studied were USA300 strain JE2 and strain SH1000. The genome sequences of both strains are known. The USA300 JE2 background represents the most prominent community-associated methicillin resistance lineage in the US, and is the strain in which the Nebraska Transposon Mutant Library is constructed [33]. Strain SH1000 is an 8325-line strain in which the defect in SigB has been corrected [39], and for many years 8325-line strains have been used as model strains in genetic studies of staphylococcal pathogenesis [36]. These strains were chosen for their significance as pathogens, well-developed knowledge of their genetics, physiology and virulence, and familiarity to the staphylococcal research community.

MH broth and LB Increase the Content of BCFAs and TSB and BHI Broth Increase the Content of SCFAs

The fatty acid compositions of strain JE2 grown in different laboratory media are shown in Fig. 3 and in more detail in S1 Table. Growth in MH broth and LB resulted in a high content

of BCFAs, 80.9% and 77.2% respectively, whereas SCFAs were 19.1% and 22.8% respectively. However, in TSB and BHI broth the BCFAs contents were lower at 51.7% and 51.5% respectively, and SCFAs were increased to 48.3 and 48.5% respectively. In MH broth anteiso odd-numbered fatty acids were the major fatty acids in the profile (59.8%), followed by even-numbered SCFAs (16.6%), iso odd-numbered fatty acids (15.8%), with iso even-numbered fatty acids making up only a minor portion (4.7%). Anteiso C15:0 was the predominant fatty acid in the membrane lipids (39%). This particular fatty acid has a significant impact on fluidizing membranes [40,41]. The anteiso fatty acids were significantly reduced in TSB-grown cells (29.3%). The major SCFAs in TSB-grown cells were C18:0 and C20:0 at 19.1% and 18.6% respectively. Overall, the fatty acid compositions were in line with many previous studies of *S. aureus* fatty acid composition [9–12], but we are unaware of previous studies that have identified this impact of medium on the proportions of BCFAs and SCFAs in the membrane.

The results of a similar series of experiments with strain SH1000 are shown in Fig. 4 and S2 Table. In strain SH1000 the BCFAs were higher than JE2 in all media- BHI 66.6%, TSB 68.5%, with particularly high contents in MH broth, 90.2%, and LB, 89%. The proportion of SCFAs was correspondingly smaller in all cases compared to strain JE2. Anteiso fatty acids were the major class of fatty acids in all media, amongst which anteiso C15:0 was present in the highest amount in all cases. However, the same phenomenon was noted where MH broth and LB encouraged a high proportion of BCFAs, low SCFAs, and TSB and BHI had the opposite effects on fatty acid composition. Two additional media were studied with this strain. Both Tryptone Broth [42] and defined medium [43] resulted in high BCFAs (80.4% and 85% respectively), and low SCFAs (19.7% and 15% respectively).

The Fatty Acid Composition of *S. aureus* Grown *ex vivo* in Serum is Radically Different to

Those of the Organism Grown in Laboratory Media

It was of interest to try and get an idea of the fatty acid composition of *S. aureus* grown *in vivo*. Strain JE2 and SH1000 were grown *ex vivo* in serum, which resulted in major changes in the fatty acid profile (Figs. 3 & 4 and S1 and S2 Tables). Total BCFAs were reduced to 37.5% in JE2 and 36.3% in SH1000; SCFAs were at 37.8% in JE2 and 32.1% in SH1000, but 25% of the fatty acid profile in the case of JE2 and 30.6% in SH1000 was accounted for by SCUFAs. Strikingly, this type of fatty acid was not present in the profile of the organism when grown in laboratory media. Interestingly, BCFAs and SCUFAs have similar effects in increasing fluidity of the membrane [4].

Carotenoid Content of Cells Grown in Different Media

Staphyloxanthin is another significant membrane component that might impact the biophysical properties of the membrane. Accordingly, the carotenoid contents of cells grown in different media were determined and the results are shown in Fig. 5. Strain SH1000 cells grown in MH broth had a much higher carotenoid content than cells grown in the other media. The pellets of cells grown in this particular media were noticeably yellow. It is possible that the carotenoid content rises to counterbalance the potentially high fluidity of MH broth-grown cells with their high content of BCFAs, specifically mainly anteiso fatty acids. MH broth (high BCFAs) and serum (high SCUFAs) - grown cells had higher carotenoid contents than TSB or BHI broth –grown cells as revealed by statistical analysis of the data (Fig. 5), which demonstrated that the carotenoid content of cells grown in MH broth was distinctly different from cells grown in other media. In strain JE2 MH broth- and serum-grown cells also had higher carotenoid contents than did cells grown in BHI broth, TSB or LB. In general, this strain was less pigmented than strain SH1000. Statistical analysis of *S. aureus* strain JE2 carotenoid content

showed that cells grown in MH broth and serum were placed in the same group, and the cells grown in BHI broth, LB and TSB were in a different group.

Membrane Fluidity of *S. aureus* Cells Grown in Different Media

The membrane fluidity of cells of strain SH1000 grown in BHI broth, LB and TSB were very similar (0.185-0.19) as shown in Fig. 6. The membranes of MH-broth and serum-grown cells, 0.25 and 0.248 were significantly less fluid than cells grown in the other media. Possibly the higher carotenoid contents of cells grown in MH broth and serum rigidify the membrane. Strain JE2 also showed a similar pattern of membrane fluidity in the different growth media (Fig. 6). The membrane fluidity of both strains was highest in cells grown in LB, consistent with the high content of BCFAs. Furthermore, in this medium there was no accompanying increase in staphyloxanthin content with its possible membrane rigidifying effect in contrast to what was observed in MH broth or serum-grown cells. Statistical analysis showed that the fluorescence polarization values of both *S. aureus* strain JE2 and SH1000 grown in MH broth and serum were significantly different from the cells grown in BHI broth, TSB and LB as indicated in Fig. 5.

Discussion

From numerous studies over the past several decades of *S. aureus* grown *in vitro* in various laboratory media it is considered that the membrane fatty acid composition of the organism is a mixture of BCFAs and SCFAs [9–12], and BCFAs have generally been found to be predominant. Through study of a range of different conventional growth media, we found that certain media encouraged a higher proportion of BCFAs than others, whereas in some media the proportion of SCFAs was increased. This may have significant physiological ramifications given the opposing effects of BCFAs and SCFAs on membrane fluidity with BCFAs fluidizing and SCFAs rigidifying the membrane [4]. However, there was a radical change in the entire fatty

acid composition when the organism was grown *ex vivo* in serum with SCUFAs appearing in the profile in significant amounts accompanied by a decrease in BCFA content.

What Determines the Balance Between BCFAs and SCFAs in Cells Grown in Laboratory Media?

MH medium leads to a high proportion of BCFAs in the staphylococcal cells, whereas growth in TSB leads to an increase in the proportion of SCFAs. MH broth (Difco) is composed of beef extract powder (2 g/l), acid digest of caseine (17.5 g/l), and soluble starch (1.5 g/l). Thus, by far the major medium component is acid digest of caseine, and this is expected to be high in free amino acids. TSB (Difco) is composed of pancreatic digest of caseine (17 g/l), enzymatic digest of soybean meal (3 g/l), dextrose (2.5 g/l), sodium chloride (5 g/l) and dipotassium phosphate (2.5 g/l). The major components then of TSB are a mixture of peptides formed by enzymatic digestion of caseine and soybean meal. Payne and Gilvarg [44] fractionated Bacto Neopeptone using gel filtration. They found that peptides with a molecular weight below 650 represented about 25% of the mixture, and free amino acids were about 1% of the entire preparation. We believe that the free amino acids from the acid digest of caseine can have a dominant effect on the fatty acid composition. Inclusion of isoleucine, leucine, or valine in the growth medium of *Listeria monocytogenes* results in large increases in fatty acids derived from the particular amino acid in question [29,45]. The BCFA content of *S. aureus* is lower in *S. aureus* grown in TSB, where pool amino acids are likely to be mainly derived from transported peptides [46], than when grown in defined medium, which probably gives rise to higher pool levels of amino acids [47] and this work. Mutants of *S. aureus* in the transporters of leucine and valine lacked odd- and even-numbered fatty acids derived from these amino acids when grown in defined medium [47].

Growth in media such as TSB and BHI broth lead to higher proportions of SCFAs than media such as MH broth, although SCUFAs were not detected. The origin of SCFAs is not clear as to whether they originate from the medium or are biosynthesized. Typically, in bacteria SCFAs are biosynthesized from acetyl CoA via the activities of FabH and the FASII system. However, acetyl CoA was a poor substrate for *S. aureus* FabH [48], whereas the enzyme had high activity for butyryl CoA, raising the possibility that butyrate is the primer for biosynthesis of SCFAs in *S. aureus*. It is also possible that SCFAs that may be present in TSB and BHI may be utilized directly for fatty acid elongation to the SCFAs in the membrane typical of growth in these media.

The Underappreciated Ability of *S. aureus* to Incorporate Host Fatty Acids from Serum

A striking finding in our paper is that *S. aureus* has the capacity to incorporate large proportions of SCFAs and SCUFAs when grown *ex vivo* in serum. Earlier reports of fatty acid composition have not reported significant amounts of SCUFAs in *S. aureus* [9–12]. Indeed, it appears that *S. aureus* lacks the genes necessary to biosynthesize unsaturated fatty acids [18]. An early report by Altenbern [49] showed that inhibition of growth by the fatty acid biosynthesis inhibitor cerulenin could be relieved by SCFAs or SCUFAs, implying *S. aureus* had the ability to incorporate preformed fatty acids. Fatty acid compositional studies of the cells were not reported though. Serum is lipid rich [50–52] and a comprehensive analysis of the human serum metabolome including lipids has recently been published [53]. BCFAs are present, if at all, in only very small amounts in serum. Bacterial pathogens typically have the ability to incorporate host-derived fatty acids thereby saving carbon and energy since fatty acids account for 95% of the energy requirement of phospholipid biosynthesis [54]. Exogenous fatty acids readily penetrate the membrane and are activated by a fatty acid kinase to produce acyl phosphate that can be utilized

by PlsY for incorporation into the 1 position of the glycerol moiety of phospholipids, or they can be converted to acyl-ACP for incorporation into the 2 position by PlsC (Fig. 2) [18].

The FASII pathway has been considered to be a promising pathway for inhibition with antimicrobial drugs. The viability of FASII as a target for drug development was challenged by Brinster *et al*. [55] especially for bacteria such as streptococci where all the lipid fatty acids could be replaced by SCFAs and SCUFAs from serum. However, Parsons *et al*. [17] showed that exogenous fatty acids could only replace about 50% of the phospholipid fatty acids in *S. aureus* and concluded that FASII remained a viable drug target in this organism.

Besides occurring in membrane phospholipids, fatty acids are present in staphyloxanthin, glycolipids and lipoteichoic acid and in lipoproteins at their N terminus in the form of an N-acyl-S-diacyl-glycerol cysteine residue and an additional acyl group amide linked to the cysteine amino group [56]. It is estimated that there are 50-70 lipoproteins in *S. aureus*, and many of them are involved in nutrient acquisition. The distribution of growth environment-derived SCFAs and SCUFAs in these lipid molecules has not yet been examined.

Changes in Staphyloxanthin in Cells Grown Under Different Conditions with Different Membrane Fatty Acid Compositions

The carotenoid staphyloxanthin is a unique *S. aureus* membrane component that affects membrane permeability, defense against reactive oxygen species, and is a potential drug target. It appeared that cells grown in media encouraging a high proportion of BCFAs or in serum resulting in high SCUFAs, both of which would be expected to increase membrane fluidity, tended to have higher staphyloxanthin contents. Cells grown in MH broth or serum had cellular membranes that were less fluid that may be attributable to the higher content of staphyloxanthin. However, this

relationship is likely to be complex in that LB-grown cells that had high BCFAs did not have high carotenoid levels, and the phenomenon is deserving of more detailed investigation. Interestingly, in the biosynthesis of staphyloxanthin, the end step involves an esterification of the glucose moiety with the carboxyl group of anteiso C15:0 by the activity of the enzyme acyltransferase CrtO [19]. It is not known whether anteiso C15:0 can be replaced by SCUFAs.

Plasticity of *S. aureus* Membrane Lipid Composition and its Possible Ramifications in Membrane Biophysics and Virulence

Given the crucial role of the biophysics of the membrane in all aspects of cell physiology, such radical changes in the membrane lipid profile can have significant but as yet undocumented impacts on critical functional properties of cells such as virulence factor production, susceptibilities to antimicrobials and tolerance of host defenses. It is important to assess the biophysical and functional properties of the membranes of the cells with such radically different fatty acid compositions.

The susceptibilities of the strains to three antibiotics designated as hydrophobic**,** oxacillin, vancomycin and rifampicin and three designated as hydrophilic, chloramphenicol, penicillin G, and tetracycline [57], were determined on TSB, BHI, MH, LB and serum agar plates by disk diffusion. There was no striking difference in antibiotic susceptibilities between the different artificial media. Zones of inhibition were markedly lower in both strains on serum agar for rifampicin, chloramphenicol, and tetracycline. However, membrane permeability studies clearly need to be done in a more simplified system such as lipid vesicles of defined fatty acid composition [58] to simplify the interpretation of any differences observed. Cells grown in serum

had higher hemolytic activity, and MH broth and serum- grown cells had lower autolytic activities than cells grown in the other media (unpublished observations).

Although BCFAs and SCUFAs both increase membrane fluidity, they do not yield cells with identical morphologies [15], or fitness for tolerating cold stress [59]. Also a *S. aureus* fatty acid auxotroph created by inactivation of acetyl coenzyme A carboxylase (*ΔaccD*) was not able to proliferate in mice, where it would have access to SCFAs and SCUFAs [60]. Due to the ability of a pathogen to adapt and undergo dramatic alterations when subjected to a host environment, there is a growing appreciation in the research community for the fact that the properties of the organism grown *in vivo* are probably very different from when it is grown *in vitro*. This distinction may have a huge impact on critical cellular attributes controlling pathogenesis and resistance to antibiotics. Expression of virulence factors is significantly different in serum-grown organisms [31], and there are global changes in gene expression when *S. aureus* is grown in blood [61]. *S. aureus* grown in serum or blood will have different membrane lipid compositions than cells grown in laboratory media and this may have a significant impact on the expression of virulence factors and pathogenesis of the organism.

The relationship between *S. aureus* and long-chain SCUFAs and SCFAs is a complex one. On one hand these fatty acids in the skin and other tissues form part of the innate defense system of the host due to their antimicrobial activities [62–64]. Very closely related structures can either be inhibitory to growth at low concentrations, or can have little effect on growth at relatively high concentrations [42,65–67]. For example, C16:1Δ6 and C16:1Δ9 are highly inhibitory whereas C18:1Δ9 and C18:0 are not inhibitory and are actually incorporated into the phospholipids by this pathogen [42].

The enzyme fatty acid kinase (Fak) responsible for incorporation of extracellular fatty acids into *S. aureus* phospholipids [18], is also a critical regulator of virulence factor expression [68], and biofilm formation [69]. Fak phosphorylates extracellular fatty acids for incorporation into *S. aureus* membrane phospholipids [18] (Fig. 2). FakA is a protein with an ATP-binding domain that interacts with FakB1 and FakB2 proteins that bind SCFAs and SCUFAs preferentially respectively. Fak activity producing acyl phosphates was proposed to be involved in the control of virulence gene expression. Interestingly FakB2 shows a high degree of specificity for C18:1Δ9, a fatty acid not produced by *S. aureus*, that may act as a sensor for the host environment through its abundance in the host [18], which is subsequently incorporated into the membrane lipids.

Additionally, fatty acids are important components of lipoproteins that contribute important microbe-associated molecular patterns that bind to Toll-like receptors and activate innate host defense mechanisms. Recently, Nguyen *et al*. [70] have shown that when *S. aureus* is fed SCUFAs they are incorporated into lipoproteins and the cells have an increased toll-like receptor 2- dependent immune stimulating activity, which enhances recognition by the immune defense system.

Concluding Remarks

We have demonstrated a hitherto poorly recognized growth environment-dependent plasticity of *S. aureus* membrane lipid composition. The balance of BCFAs and SCFAs was affected significantly by the variations in laboratory medium in which the organism grew. SCUFAs became a major membrane fatty acid component when the organism was grown in serum. These findings speak to the properties of pathogens grown *in vitro* versus *in vivo*. In 1960 Garber [71] considered the host as the growth medium and the importance of the properties of the pathogen

at the site of infection. There has been a renewed appreciation of this in recent years [72]. Massey *et al*. [73] showed that *S. aureus* grown in peritoneal dialysate acquired a protein coat. Krismer *et al*. [74] devised a synthetic nasal secretion medium for growth of *S. aureus*. However, Chaves Moreno *et al*. [75] determined the *in vivo* metatranscriptome of *S. aureus* by RNAseq analysis of RNA isolated from the anterior nares of documented *S. aureus* carriers. *In vitro* transcriptomes did not mimic *in vivo* transcriptomes. Citterio *et al*. [76] reported that the activities of antimicrobial peptides and antibiotics were enhanced against various pathogenic bacteria by supplementation of the media with blood plasma to mimic *in vivo* conditions.

S. aureus may be the most versatile of all pathogens causing diseases ranging from superficial skin infections to deep seated disseminated diseases of various organs and tissues. The organism forms biofilms on tissues, intravenous catheters and prosthetic devices. *S. aureus* can thrive in multiple heterogenous environments. We propose that the nutritional environment is the main determinant of membrane fatty acid composition. If SCUFAs are present in the environment these will be preferentially incorporated into the lipids to a tolerated extent, although there appears to be a requirement for a significant proportion of biosynthesized anteiso odd-numbered fatty acids. It is sobering to realize that the vast majority of studies of staphylococcal biology utilizing organisms grown in artificial media have been carried out with cells lacking SCUFAs in their membrane.

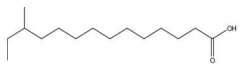
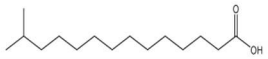
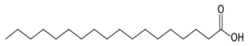
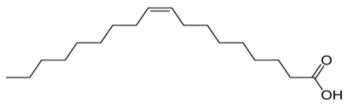
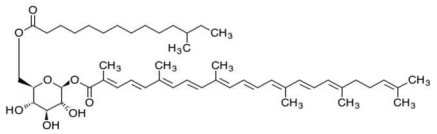
BCFA	Anteiso C15:0	
	Iso C15:0	
SCFA	C18:0 (Stearic acid)	
SCUFA	C18:1Δ9 (Oleic acid)	
Staphyloxanthin	β-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate)	

Fig 1. Structures of major fatty acids and staphyloxanthin of the *S. aureus* cell membrane.

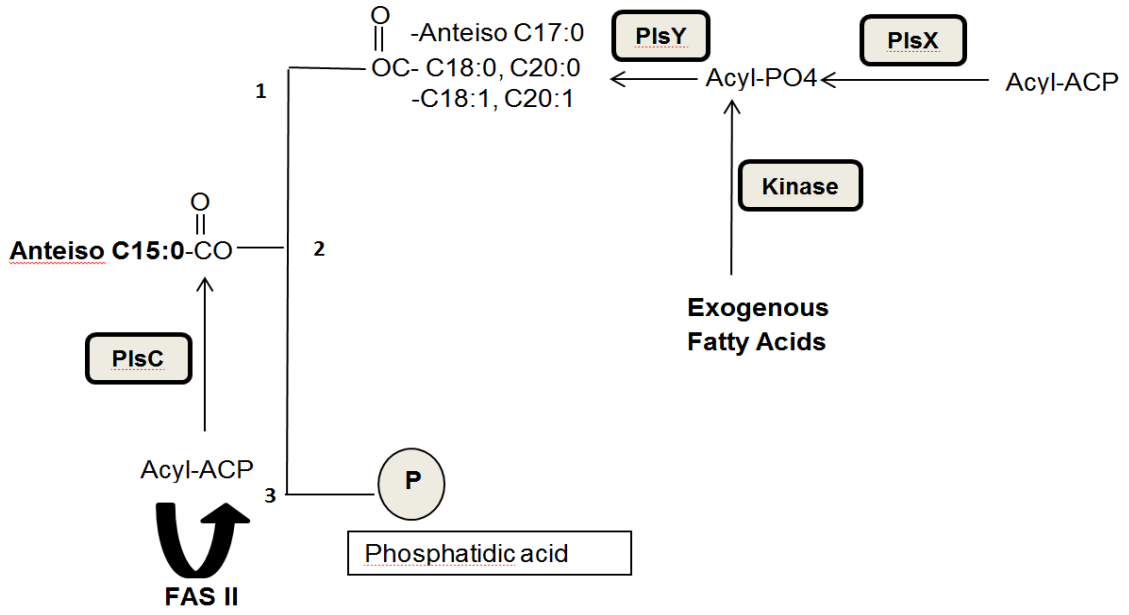


Fig 2. Pathway of phospholipid biosynthesis and the incorporation of exogenous and endogenous fatty acids in *S. aureus.*

Phosphatidic acid (PtdOH), the universal precursor of phospholipids, is synthesized by the stepwise acylation of *sn*-glycerol-3-phosphate first by PlsY that transfers a fatty acid to the 1-position from acyl phosphate. The 2-position is then acylated by PlsC utilizing acyl-ACP. Acyl-ACP is produced by the FASII pathway and PlsX catalyses the interconversion of acyl-ACP and acyl phosphate. Exogenous fatty acids readily penetrate the membrane and are activated by a fatty acid kinase (FakB1 for SCFAs and FakB2 for SCUFAs) to produce acyl phosphate that can be utilized by PlsY, or that can be converted to acyl-ACP for incorporation into the 2-position by PlsC. Exogenous fatty acids can also be elongated by the FASII pathway. Figure modified from Parsons *et al*. [18].

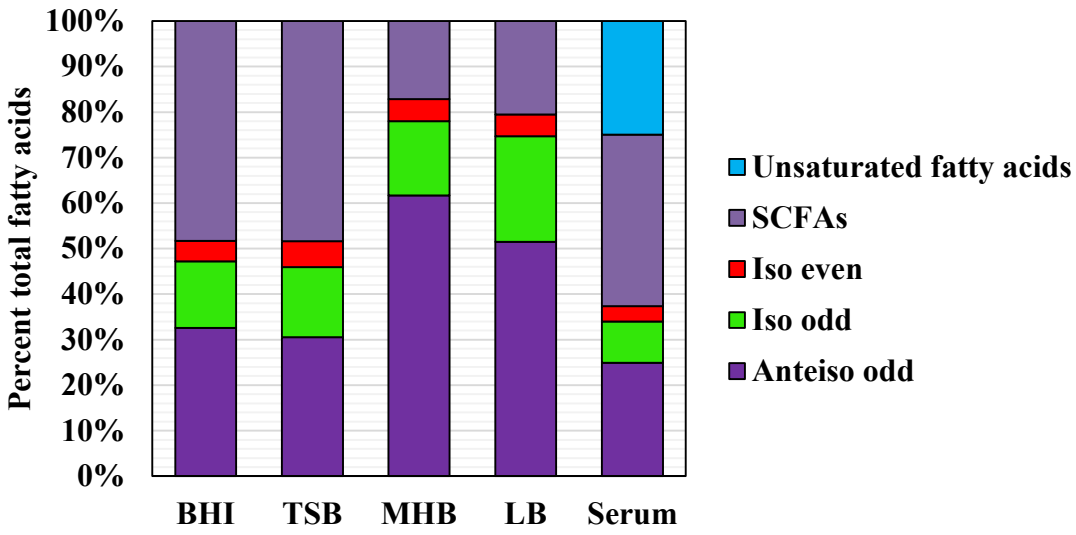


Fig. 3. Membrane fatty acid composition of *S. aureus* strain JE2 cells grown in different media.

Membrane fatty acid composition of log phase *S. aureus* strain JE2 cells grown in BHI, TSB, MHB, LB and fetal bovine serum were summarized into the various common classes of fatty acids. Figure shows representative data from at least three independent experiments.

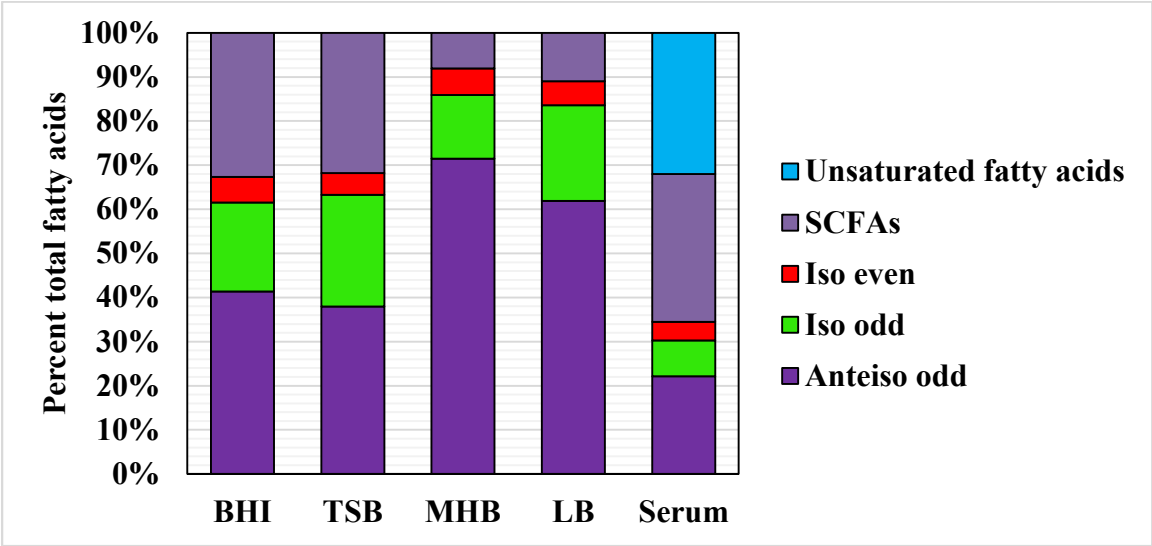


Fig. 4. Membrane fatty acid composition of *S. aureus* strain SH1000 cells grown in different media.

Membrane fatty acid composition of log phase *S. aureus* strain SH1000 cells grown in BHI, TSB, MH broth, LB and fetal bovine serum were summarized into the various common classes of fatty acids. Figure shows representative data from at least three independent experiments.

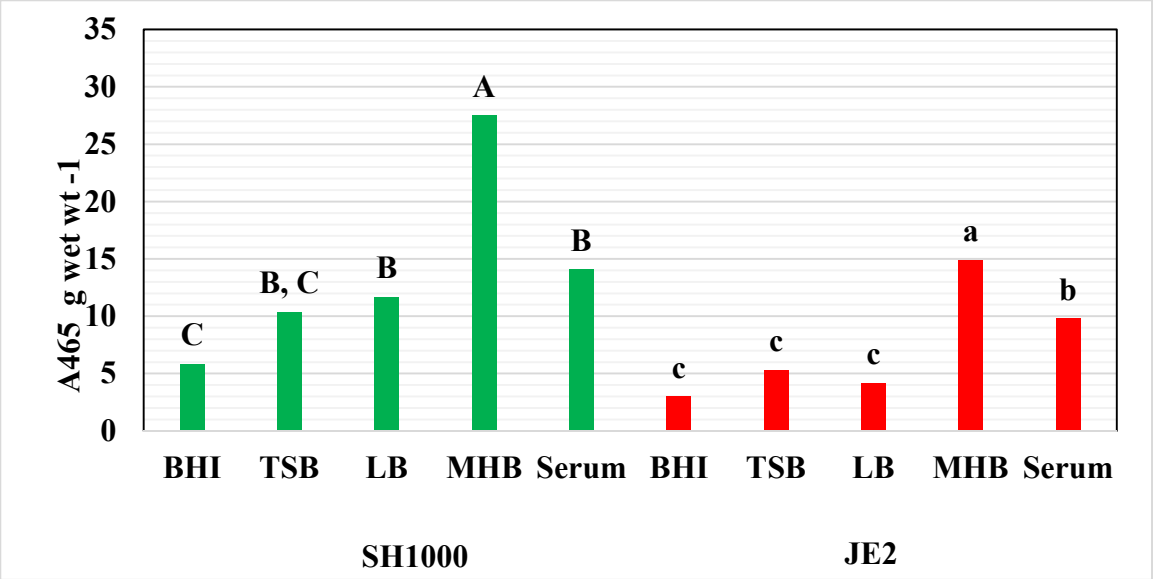


Fig 5. Influence of growth environment on the carotenoid content of *S. aureus.*

The strains, JE2 (red columns) and SH1000 (green columns), were grown in different growth media and the carotenoid content was estimated after extraction by warm methanol. Different letters indicate significant differences in the carotenoid content.

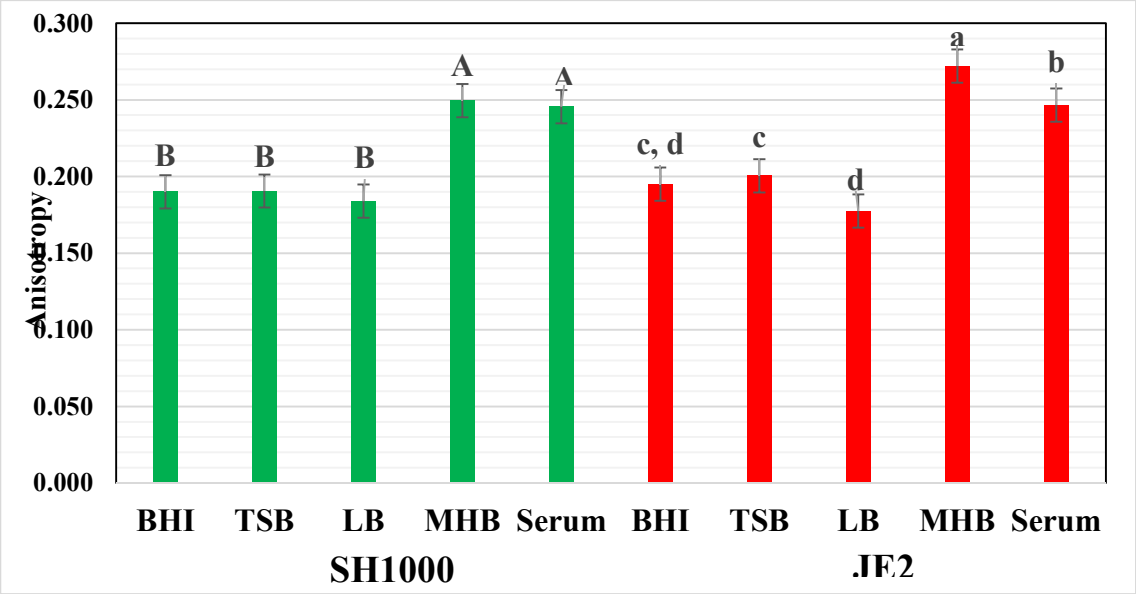


Fig 6. Influence of growth environment on the membrane fluidity of *S. aureus* cells.

The strains, JE2 (red columns) and SH1000 (green columns), were grown in the different media to mid exponential phase and membrane anisotropy was measured by fluorescence polarization. Different letters indicate statistically significant differences in the fluorescence polarization values.

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Supporting information

S1 Table. The membrane fatty acid profile of *S. aureus* strain JE2 grown in various conventional media and in serum

% (wt/wt) of total fatty acids

Membrane fatty acids		BHI	TSB	MHB	LB	Serum
Anteiso odd	C15:0	28.5	26.9	39	36.5	21
	C17:0	2.6	2.4	15	11	4
	C19:0	ND	ND	5.8	2.4	ND
Iso odd	C15:0	12.2	12.8	7.7	13.3	7.1
	C17:0	1.8	2	4.9	6.6	2
	C19:0	ND	ND	3.2	2.6	0
Iso even	C14:0	2.9	3.7	1	1.4	1.7
	C16:0	1.4	1.8	2.1	2.2	1.7
	C18:0	ND	ND	1.6	1	ND
Straight even	C14:0	3.3	2.3	1.1	0	1.7
	C16:0	7.9	6.4	1.8	3	16.4
	C18:0	21.5	19.1	6.5	10	13.3
	C20:0	13.4	18.6	7.2	6.9	4.8
Unsaturated fatty acids	C16:1Δ9	ND	ND	ND	ND	1.7
	C18:1Δ9	ND	ND	ND	ND	16
	C18:1Δ7	ND	ND	ND	ND	4.2
	C20:1Δ9	ND	ND	ND	ND	2.1
	C20:4Δ6,9,12,15	ND	ND	ND	ND	1
BCFAs		49.4	49.6	80.3	77	37.5
SCFAs		46.1	46.4	16.6	19.9	37.8
SCUFAs		ND	ND	ND	ND	25

ND- Not detected

S2 Table. The membrane fatty acid composition of *S. aureus* strain SH1000 grown in various conventional media and in serum

% (wt/wt) of total fatty acid

Membrane fatty acids		BHI	TSB	MHB	LB	Serum
Anteiso odd	C15:0	33.6	31.6	43.7	42	18.2
	C17:0	6.4	6	20.7	16.8	3
	C19:0	1	ND	5.7	3.1	ND
Iso odd	C15:0	15.7	18.7	6.8	12.2	6
	C17:0	4.3	5.2	5.1	7.2	1.8
	C19:0	ND	1.2	2.3	2.3	ND
Iso even	C14:0	2.6	2.2	1.2	1.3	2.4
	C16:0	3.1	2.7	3.1	2.9	1.6
	C19:0	ND	ND	1.6	1.2	ND
Straight even	C14:0	2.7	2	ND	ND	1.1
	C16:0	8.1	7.8	1.4	2.4	13.2
	C18:0	15.2	15.4	4.2	6	12.1
	C20:0	6.4	6.3	2.3	2.6	4.4
Unsaturated fatty acids	C16:1Δ9	ND	ND	ND	ND	1.5
	C18:1Δ9	ND	ND	ND	ND	15.4
	C18:1Δ7	ND	ND	ND	ND	6.4
	C20:1Δ9	ND	ND	ND	ND	5
	C20:4Δ6,9,12,15	ND	ND	ND	ND	2.3
BCFAs		66.7	67.6	90.2	89	33
SCFAs		32.4	31.5	7.9	11	32.1
SCUFAs		ND	ND	ND	ND	30.6

ND- Not detected

CHAPTER III

SUMMARY

The significance of a membrane homeostasis, regulated by the composition of the lipid bilayer for survival and growth of a cell is huge. The phospholipid acyl chains influence membrane viscosity/fluidity, and impact the ability of bacteria to adapt to changing environments, the passive permeability of hydrophobic molecules, active transport, and the function of membrane-associated proteins. Additionally, membrane fatty acid composition has a major influence on bacterial pathogenesis, and broader aspects of bacterial physiology. Hence in recent times there has been increased research to better understand the lipid biosynthetic pathways and their regulations in bacteria pathogens to support efforts in translational medicine attempting to target these critical processes with novel antimicrobials.

This current study highlights the dynamics of membrane lipid composition in Gram positive bacterial pathogens and the underlying regulation and molecular networks. It also draws attention to the fact that changes in the external environment have significant consequences on membrane fatty acid profile and biophysical properties. In *L. monocytogenes* this may involve a native metabolic pathway to form novel fatty acyl chains in the phospholipid bilayer from synthetic precursors by non-native promiscuity to support the membrane integrity for proper survival; thereby establishing an alternative pathway for lipid utilization. The membrane fluidity of this pathogen is one of the critical parameters that govern its unique survival at very low temperatures making it a leading cause of food borne infections recently. The ability to use

synthetic molecules, which are common food additives, to produce novel fatty acids having surprisingly correct biophysical properties to help restoring desired membrane homeostasis in this pathogen is a remarkable find. It helps us realize the efficiency of the bacterial cell to modulate existing pathways for proper maintenance of critical membrane parameters. Future studies on the functional properties of such unique lipid bilayers may prove beneficial to understand the pathogen physiology better and also provide advantage in developing novel and efficient means of control.

In *S. aureus* the membrane composition was proved to be highly plastic depending on the medium of growth; a finding that makes us question- What is the fatty acid composition that gives *S. aureus* the best fitness? The proportions and components of the fatty acids change drastically from *in vitro* to *in vivo* models, where host derived fatty acids seem to play a major role in modulating lipid profile. In *S. aureus* increased membrane fluidity has been associated with decreased susceptibility to membrane-active peptides and organic solvents. Given that MH medium is the standard medium for antimicrobial MIC determinations it is possible that certain compounds have higher MICs in this medium, and strains may be erroneously designated as resistant or decreased susceptibility. Moreover, the incorporation of SCUFAs in the membrane when grown in serum may have drastic yet unknown consequences on such susceptibilities. This may be particularly significant in VISA and daptomycin-decreased susceptibility strains, which typically only show small increases in MICs compared to susceptible strains. Hence it only seems fair due to the broad lipid plasticity of this pathogen, to suggest a medium of growth which more closely mirrors the host environment to better understand the host pathogen interactions and its significance in the physiology of this pathogen.